Development of a Method for Determination of the delta-9-Tetrahydrocannabinol Metabolite in Urine Using an Online-Solid Phase Extraction Prior to Liquid Chromatography-Mass Spectrometry Analysis

Waranyu Nachiangtai

A Thesis Submitted in Partial Fulfillment of Requirements for

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May 2024

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ABSTRACT

Delta-9-tetrahydrocannobinol (THC) is the bioactive compound in marijuana (*Cannabis sativa* L.) that can be used for medical purposes at low concentrations. THC is metabolized in the liver and transformed to 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (11-nor-Δ9-THC-COOH), which is excreted in urine. Therefore, this study aims to develop a sensitive analytical method for the determination of the THC metabolite in urine at trace level. Online-solid phase extraction (HyperSep Retain PEP Online-SPE column) was employed to extract of 11-nor-Δ9-THC-COOH in urine before analysis by liquid chromatography-mass spectrometry (LC-MS). The online-SPE is a simple and rapid that reduces the preparation steps by passing the sample through the SPE cartridge and then eluting with appropriated solvent before separating by LC-MS. This method demonstrated the detection limit and quantitative limit at 0.20 ng mL^{-1} and 1.00 ng mL^{-1}, respectively. The linearity range was 1.00-100.00 ng mL^{-1}, with a correlation coefficient (r) greater than 0.9995. According to the SWGTOX guidelines, the accuracy value (%bias) and precision value of the developed method were considered within the acceptable criteria (± 20%). The percentage of recovery was observed in the range 99.16–101.60%. The dilution factor of 20 showed the %bias in the range of -6.24–2.74%. The stability of the sample was maintained for 24 hours in the auto-sampler, and reanalysis stability was maintained for 72 hours. Therefore, this method is accurate, precise and appropriate for analyzing 11-nor-Δ9-THC-COOH metabolite in urine sample at low-level concentrations.

Keyword : delta-9-tetrahydrocannobinol, marijuana, urine, online-solid phase extraction

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Waranyu Nachiangtai

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

1.1 Problems and provenance

Cannabinoids and delta-9-tetrahydrocannabinol (THC) are commonly presented in *Cannabis sativa L.*, THC is the most psychologically active component [1]. Cannabis can be consumed by smoking, eating or mixing with food. It has been used in Thai traditional medicine for very long time. It contains about five hundred natural compounds. One hundred compounds are classified as cannabinoids found only in cannabis. Mostly, cannabinoids are found in unfertilized female flowers. Flower is an important part to develop medical products. The important substance and main active ingredient are delta-9-tetrahy-drocannobinol (THC). Cannabis are also known as marijuana, has a number of health benefits for humans, such as treating glaucoma, controlling epileptic seizures, and stopping cancer from spreading [2]. Cannabidiol (CBD), a further well-known cannabinoid, is regarded as non-intoxicating with the other effects including anticonvulsive, anti-inflammatory, mildly sedative, and anxiolytic properties [3]. CBD was mainly excreted through the feces in an unchanged form. THC substances entering the body was destroyed in the liver and changed to 11-Nor-delta 9-tetrahydrocannabinol-9-carboxylic acid (11-Nor-Δ9-THC -COOH or 9-Carboxy-THC). Then it was excreted through the urine in conjugate form. It can be detected THC in urine for up to 30 days because some THC was accumulated in the body fat tissue (Fat Tissue) and was gradually excreted in urine or feces in the form of free acid or glucuronide conjugate [4, 5].

According to the Narcotics Act of Thailand (No. 7) B.E. 2562 (2019), marijuana is only used for medicinal and research purposes. Under the guidance of a licensed physician, cannabis extracts can be prescribed [6]. At present, cannabis has been developed as a variety of therapeutic medicines or products to treatment the different patients [7]. Cannabis products were utilized to treat patients receiving palliative care, end-state cancer, Parkinson's disease, Alzheimer's disease, Generalized anxiety disorders, other demyelinating diseases such as neuromyelitis optica and autoimmune encephalitis [8]. An announcement of the Ministry of Public Health

specifying the name of narcotics in category 5, B.E. 2565 (2022) identified the extraction containing delta-9-tetrahydrocannabinol not more than 0.2 percent by weight is not a drug of abuse [9].

The Department of Medical Sciences, Ministry of Public Health as a drug testing facility has established the concentration of cannabis active substances or metabolites (according to the act to use the Narcotics Act B.E. 2564 (2021) in the urine at 50 ng/mL or more to judge criteria for proving that he or she is used cannabis as a drug addict. This concentration (50 ng/mL) is different from the medical marijuana proposed. The normal method cannot be measured cannabis metabolites excreted in urine at this concentration. Moreover, there is currently no method for detecting low levels of cannabis and/or cannabis metabolites. Therefore, this research work was developed an online LC-MS method for analyzing the amount of marijuana in the urine at low-level concentrations to coverage for patients who was used marijuana for medical proposed. In this regard, LC-MS technique can separate and detect metabolites of marijuana at ng/mL. This technique is also high sensitivity and high specificity to support and expand the capacity of laboratories to provide medical cannabis testing services according to the policy of the Ministry of Public Health on the use of medical marijuana. Urine samples are easy to collect compared with other biomaterials. It can be sampled in large quantities resulting in sufficient samples to be used for analysis. Moreover, drug residues in urine can be detected within 3-30 days [3, 5].

1.2 Objectives

1.2.1) To develop a method for the determination of Δ9-tetrahydrocannabinol metabolite using an online-solid phase extraction prior to liquid chromatography-mass spectrometry analysis

1.2.2) To apply the developed method for the determination of Δ9-tetrahydro cannabinol metabolite in real urine samples

1.3 Scope of this work

1.3.1) Investigation of the parameters for protein precipitation such as temperature and time of incubation, speed and time of centrifugation

1.3.2) Investigation on the separation conditions for the determination of Δ9-tetrahydrocannabinol metabolite by liquid chromatography-mass spectrometry such as the ratio of mobile phase

1.3.3) Optimization conditions for online SPE

1.3.4) Investigation of analytical performance and method validation in order to evaluate the specificity, carryover, limit of detection, limit of quantitation, linearity and range, accuracy, precision, recovery, matrix effect, dilution integrity and stability

1.3.5) Study of statistical data with One-way ANOVA for determining the Inter-day precision (reproducibility)

1.3.6) Study of the sample preparation for the determination of Δ9-tetrahydro cannabinol metabolite and apply to a real urine sample

1.4 Benefit of research

1.4.1) The proposed method was provided high accuracy, high precision, speed, simple extraction method and cost-effective method for the determination of Δ9-tetrahydrocannabinol metabolite in urine using an online-solid phase extraction prior to liquid chromatography-mass spectrometry analysis.

1.4.2) This developed method can apply to determine of Δ9-tetrahydro cannabinol metabolite in real urine samples.

1.5 Venue of the study

1.5.1) Narcotics laboratory, Regional Medical Sciences Center 8 Udonthani

1.5.2) Department of Chemistry, Faculty of Science, Mahasarakham University

CHAPTER II
LITERATURES REVIEW

2.1 General history of cannabis

Cannabis (in the form of marijuana and hashish) is the most commonly used illicit drug through the worldwide [10]. It is traditionally consumed by smoking, eating, or drinking in the form of infusions. The smoking of cannabis is the most harmful method for consumption [11]. Cannabis is one of the oldest known medicinal plants. Cannabis is described in ancient herbal medicine manuals. There is archaeological evidence that the plant spread from Asia to Africa to the Middle East and extended to Europe in the later 500 B.C. Cannabis was used extensively for industrial purposes. It was an important part of early transportation when processed into hemp fiber. Historically, the therapeutic used of cannabis was first introduced in Europe around 1840 by an Irish physician named William O'Shaughnessy. He has observed the widespread therapeutic used of cannabis in India. Over the next decade, cannabis was briefly popularized in Europe and America, with more than ten different forms of cannabis preparations available at the time. These cannabis products are recommended for symptoms such as menstrual cramps, asthma, cough, insomnia, labor pains, migraines, strep throat, and in cases of cessation of opioid use. At that time, there were no pharmaceutical quality control tools. Patients often receive doses that are too small to see no effect or get too much until side effects occur. These deficiencies have led to the therapeutic use of marijuana largely being replaced by opioid-derived drugs such as codeine and morphine, thus gradually disappearing from Western pharmacopoeia [12]. In the early 1960s, the main bioactive cannabinoids of THC and CBD, were discovered in the cannabis plant. Cannabinoid receptors are discovered in the human body in 1990. This was a pivotal moment in the discovery of the cannabis plant's key therapeutic compounds, and confirmed the biological efficacy of cannabis action. Since then, clinical trials have shown that cannabis has the potential to be used in the treatment of certain ailments [13, 14].

2.1.1) Botanical characteristics of cannabis

Cannabis (Marijuana) is an herbaceous plant belonging to the family Cannabidaceae in the genus *Cannabis*. There are two subspecies that are predominant in the genus, namely *Cannabis sativa* L. Subsp. *Indica*, or cannabis, and *Cannabis sativa L. Subsp. Sativa*, or hemp [13]. They were very similar external morphology. In general, cannabis stems are green but not very tall compared to hemp. Height is not more than 2 meters. The green leaves are arranged with 5-7 lobes, which are arranged close together [15]. Bloom at the age of about 3 months and the inflorescences are very rubbery. The content of THC is more than 1%, while CBD is less than 2%. It is commonly extracted and used in medicine. Hemp generally has a taller stem than cannabis (more than 2 meters tall). The light green leaves have about 7-11 lobes, with the leaves arranged quite clearly. Hemp will bloom at the age of more than 4 months. The inflorescences are not very resinous. The content of THC is less than 1%. CBD is more than 2%. It is often used in textiles, paper making, extract seeds for the food and cosmetics industry. The flower characteristics of cannabis are found in Thailand. They are male and female on different trees. Sometimes both male and female flowers may be found on the same plant [13, 16, 17].

Both plant subspecies were also found to contain essential cannabinoids, which consist of three compounds: delta-9-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD) [18]. THC is a stimulant, depressant, and hallucinogenic. CBN is a decomposed substance of THC when exposed to light or oxidized. It is a mild neuroprotective effect and is rarely found in fresh plants. CBD is a substance that counteracts the action of THC. It is currently studied to bring this substance for medical used in many countries around the world, including Thailand. Because CBD can reduce nausea and vomiting, reduces inflammation and swelling of wounds or tumors suppresses growing cancer cells, suppresses spasms or spasms and can create immunity in the nervous system. THC is a neurological effect that gives a feeling of passing through, relaxes, enchanted or that many people use and feel drunk. It is also the one that makes the sensory system work better and stimulates appetite [12].

2.1.2) Chemical composition of cannabis

More than 500 chemical compounds are produced from the cannabis plant. Among these, at least 100 compounds are the only cannabinoids presented in the plant. Cannabinoids derived from plants call phytocannabinoids [13]. These substances are chemically similar to the endocannabinoids that the body produces. It can bind to cannabinoid receptors in the endocannabinoid system. The secretion of neurotransmitters such as dopamine as well as endocannabinoids was produced by the body itself. The main of phytocannabinoids are delta-9-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD) [19, 20].

Delta-9-tetrahydrocannabinol (THC) is a chemical formula $C_{21}H_{30}O_2$. The molecular weight is 314.5. It is a viscous oil, water-insoluble, but dissolves in oil, alcohol, chloroform and acetone. It is destroyed by heat (pyrolysis). The chemical structure of THC is presented in Figure 1.

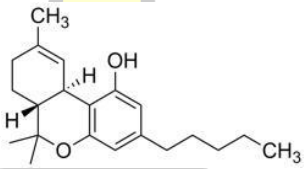

Figure 1 Chemical structure of THC.

Cannabinol (CBN) is a chemical formula $C_{21}H_{26}O_2$. The molecular weight is 310.4. It is insoluble in water, but soluble in methanol, ethanol and alkaline solutions. The chemical structure of CBN is shown in Figure 2.

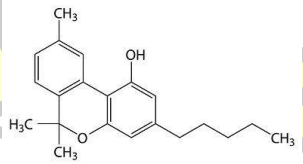

Figure 2 Chemical structure of CBN.

Cannabidiol (CBD) is a chemical formula $C_{21}H_{30}O_2$. The molecular weight is 314.5. It is a light-yellow crystal or resin (resin). It is insoluble in water, but soluble in ethanol, methanol and chloroform. The structure of CBD is demonstrated in Figure 3.

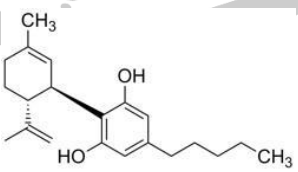

Figure 3 Chemical structure of CBD.

2.1.3) Pharmacology and pharmacokinetics of cannabis

The most studied phytocannabinoids are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is a psychoactive substance but CBD is not psychoactive [20].

THC is the most well-known cannabinoid. There are many medicinal properties of cannabis. This may include psychotropic, immunosuppressive/immunomodulation, cardiovascular, appetite stimulant, analgesic, anti-emetic and reducing nausea as well as vomiting. CBN is a product of aged THC. It has been demonstrated possible anticonvulsant and anti-inflammatory effects [21, 22].

CBD is another important cannabinoid with medicinal properties; however, it doesn't cause psychiatric conditions. This cannabinoid will not cause toxicity. This may include anticonvulsants, anti-inflammatory, anti-psychotic, analgesic, anti-anxiety, anti-arthritic and immunosuppressive [21, 22].

Cannabinoids in humans called endogenous cannabinoids, are secreted to regulate various processes in the body by binding to them. The cannabinoid receptor (CB) is composed of two receptors. The CB1 receptor found in the central nervous system and the CB2 receptor found in the peripheral nervous system and central nervous system. Both CB1 and CB2 receptors can bind to endogenous cannabinoids and exogenous cannabinoids [23-25].

CB1 receptors are found in the brain. CB1 receptors are associated with movement, perception and sensation but rarely in the brain stem. CB1 receptors are believed to involve in the psychological effects of cannabis users at the same presence of a small amount of CB1 in the brain stem. Most cannabis users do not develop a coma and respiratory depression. CB2 receptors are found mainly in the peripheral nervous system and play a role in the immune response.

The pharmacokinetics of endocannabinoids (2-arachidonolglycerol, 2-AG) shown in Figure 4, is built from diacylglycerol precursors within the cell. The action of the enzyme DAG lipase acts to convert diacylglycerol to 2-arachidonolglycerol. When endocannabinoids were binding to CB1 receptors at presynaptic neurons, G protein was inhibited adenylyl cyclase activity at presynaptic neurons resulting in a reduction of cAMP production, and calcium influx into presynaptic neurons. More potassium flows out to the presynaptic neurons causing a state of hyperpolarization,

resulting in decreasing in the secretion of neurotransmitters neurotransmitter from the presynaptic cell to the synaptic cleft. In addition, when endocannabinoids are bound to the CB1 receptor. Then activates the G protein is turn activates the activity of MPAK in performs the main functions of cells in differentiation, proliferation and cell death. Endocannabinoids binding with the CB1 receptor will move into postsynaptic neurons and are destroyed by enzyme activity monoacylglycerol lipase (MAG lipase) [7]. The mechanism of action of endocannabinoids was presented in Figure 4.

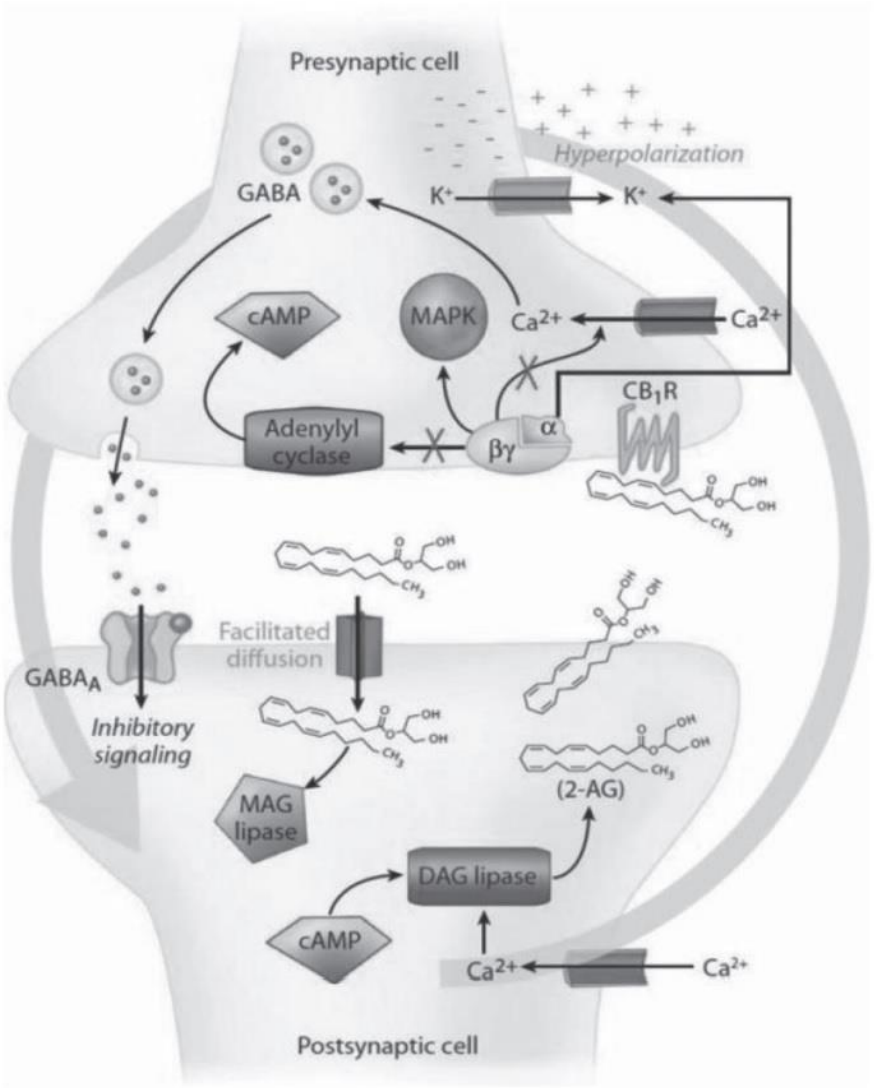

Figure 4 The mechanism of action of endocannabinoids [7].

The metabolism of THC was occurred in the liver through the action of CYP2C9 and CYP3A4. Hydroxylation is the main active metabolite in human urine, namely 11-hydroxy delta-9-THC. This is further oxidized to the inactive carboxylic acid metabolite followed by oxidation to the inactive metabolite, providing 11-nor-delta-9-THC carboxylic acid, which undergoes a phase II conjugation reaction with glucuronic acid and is excreted in the urine [24, 26, 27]. Figure 5 shows the metabolism of THC in human.

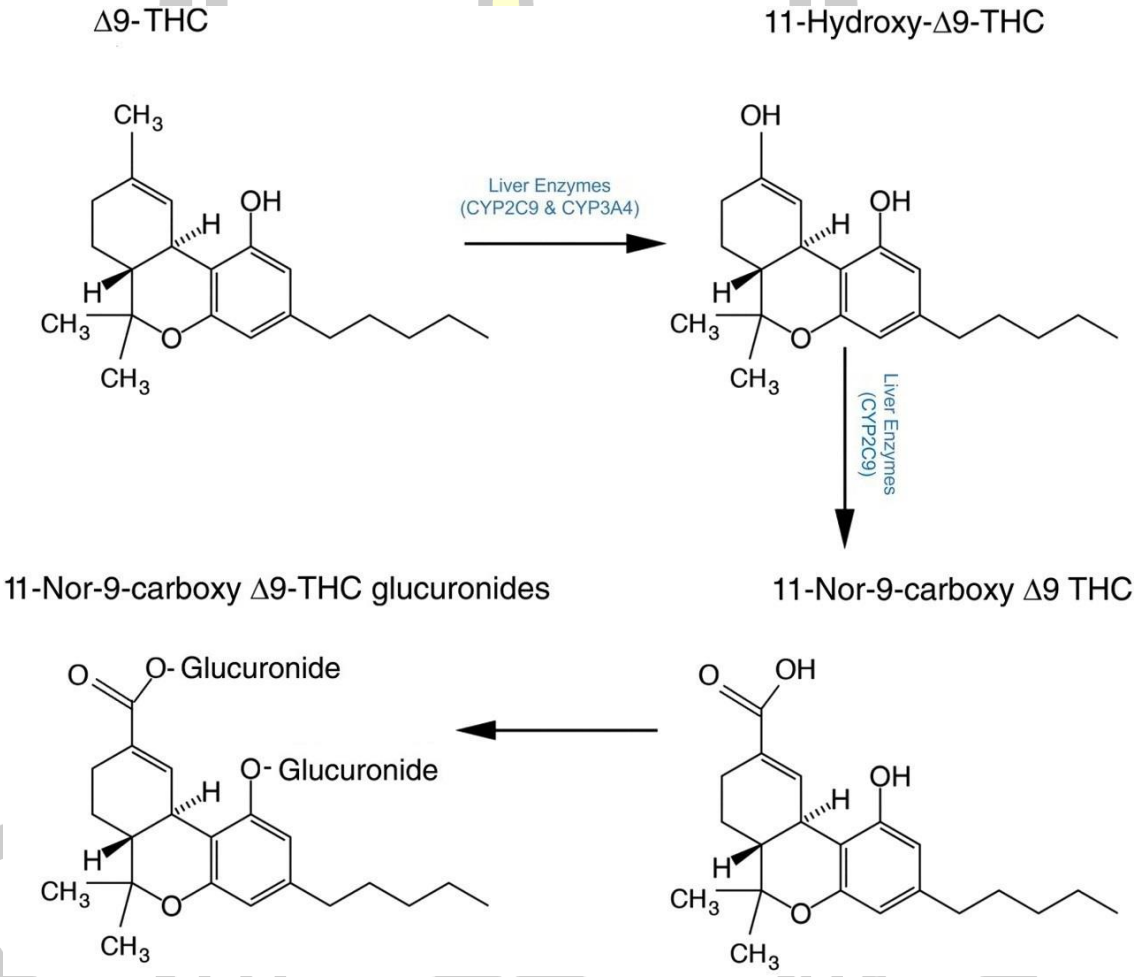

Figure 5 The metabolism of THC [27].

2.1.4) Cannabis in Thai medicine

Explanation of Phra O-sot Phra Narai's textbook mentions cannabis in many issues. Cannabis was used in small amounts such as those used in this formulation. Thai people have known to use it in cooking since ancient times to improve food more delicious and eat more rice as an appetite suppressant. Nowadays, it can be scientifically proven in cannabis. There are many substances that make eating food more flavorful and eaten in large quantities [8, 28]. A list of cannabis in Thai medicine and indications for symptom relief is presented in Table 1.

Table 1 List of cannabis in Thai medicine.

Name (in Thai)	Indication
ยาอัคคินีวคณะ	Relieve nausea and vomiting.
ยาสุขไสยาศน	Helps with sleep and appetite.
ยาแก้ลมเนาวนารีวาโย	Relieve sudden pain at the tips of the hands and toes, chest tightness, neck stiffness, inability to turn the neck.
ยาน้ำมันสนั่นไตรภพ	Relieve the wind condensed in the lower abdomen.
ยาแก้ลมขึ้นเบื้องสูง	Solve wind diseases that cause headaches, red eyes, blurry ears, tinnitus, fatigue, swings, etc.
ยาไฟอาวุธ	Relieve colic and abdominal pain.
ยาแก้นอนไม่หลับ /ยาแก้ไข้ผอมเหลือง	Solve insomnia, fever, trembling and fatigue.
ยาแก้สันฑฆาต กล่อนแห้ง	Relieve constipation, aches and pains all over the body, numb hands and feet, headache, dizziness, heartburn and chest tightness.
ยาอัมฤตโอสถ	Relieve weak hands and feet.
ยาอไภยสาล	Relieve colic.
ยาแก้ลมแก้เส้น	Relieve numbness and weakness of hands and feet.
ยาแก้โรคจิต	Solve diseases that cause anxiety, stress, insomnia.
ยาไพสาลี	Relieve cough with phlegm.
ยาทาริดสีดวงทวารหนักและโรคผิวหนัง	Cure hemorrhoids and cure skin disease.
ยาทำลายพระสุเมร	Solve colic, dizziness and headache.
ยาทัพยาธิคุณ	Relieve colic, pain, eating food, not knowing the taste and insomnia.

2.1.5) Cannabis in modern medicine

The used of cannabis extracts in medicine efficiency and safety are important considerations for patients. Cannabis extracts (not lab-synthesized) used in medicine can be divided into 3 groups [29].

1. Cannabis extract for therapeutic benefits which have clear supporting academic information.

- Nausea and vomiting in patients receiving chemotherapy.
- Pediatric epilepsy and drug-resistant epilepsy.
- Muscle spasms in patients with degenerative nerve sheaths.
- Neuralgia where other treatments have failed.

2. Cannabis extracts may be useful in diet control, which should have supported academic information or additional research on safety and effectiveness issues to support adoption such as :

- Parkinson's disease.
- Alzheimer's disease.
- Multiple sclerosis.
- Generalized anxiety disorder (GAD).
- Patients requiring palliative care.
- Terminal cancer patients.

3. Cannabis extracts have therapeutic benefits, but there is a lack of clear evidence to support their safety and effectiveness, which must be studied in vitro and in experimental animals before bringing it into human research studies, such as the treatment of different types of cancer.

2.2 Method for the determination of Δ9-tetrahydrocannabinol metabolite in urine

Vlase, Laurian *et al.*,[11] was studied high-throughput toxicological analysis of Δ9-THC in suspect plant material and 11-nor-9-carboxy-Δ9-THC in human urine by LC/MS/MS 1100 series Agilent Technologies and Agilent Ion Trap Detector 1100 SL. The plant samples (0.2 g) were extracted in methanol and diluted before the analysis. The urine samples were heated for 30 min at 80°C with 85% phosphoric acid and centrifuged before the analysis. The results, LLOQ were 2.0 ng/mL for THC, and 0.5 ng/mL for THC-COOH, respectively. The advantages of this method are

inexpensive and less time analysis. The sample was used only 0.2 g for plant samples and 1.0 mL for urine.

Frei, P. *et al.*,[3] was analyzed Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC (OH-THC), 11-nor-9-carboxy-THC (THC-COOH), Cannabidiol (CBD), and Cannabinol (CBN) in whole blood and urine via on-line SPE by derivatization with N-methyl-N-(trimethylsilyl) trifluoracetamid (MSTFA), coupled to gas chromatography tandem mass spectrometry by Thermo Fisher Scientific. The limits of detection in whole blood and urine were 0.15 ng/mL for THC, OH-THC and CBD, 0.1 ng/mL for CBN, and 1.0 ng/mL for THC-COOH, respectively. The limits of quantification in whole blood and urine were 0.3 ng/mL for THC, OH-THC and CBD, 0.2 ng/mL for CBN, and 3.0 ng/mL for THC-COOH, respectively. Sample was used only 0.25 mL. The method enables a throughput of approximately 30 samples per 24 h with only minimal manual labor involved.

Konig, S. *et al.*,[30] was determined Δ9-tetrahydrocannabinol (THC) and two major metabolites in human peripheral blood via on-line SPE coupled to liquid chromatography tandem mass spectrometry by Phenomenex and Sciex. This method was sample preparation with protein precipitation by acetonitrile. The limits of detection were obtained 0.18 ng/mL for THC, 0.18 ng/mL of 11-OH-THC and 0.85 ng/mL for THC-COOH, respectively. For the limits of quantification were obtained 0.44 ng/mL for THC, 0.45 ng/mL for 11-OH-THC, and 2.0 ng/mL for THC-COOH, respectively. Sample was used only 200 µL. This method was not hydrolysis because the hydrolysis will cause high pH.

De Souza Eller, Sarah Carobini Werner *et al.*,[31] was quantified 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in urine samples by combination of hollow fiber-liquid phase microextraction and gas chromatography–mass spectrometry. The limit of detection was 1.5 ng/mL. This method is simplicity, rapidness and inexpensiveness.

Golding Fraga, S. *et al.*,[32] was investigated stability of cannabinoid compounds in urine samples at three temperatures: room temperature (20-25°C), refrigeration (4°C), and freezing temperature (-18°C). Important losses of 8.0 ± 10.6, 15.8 ± 4.2, and 19.6 ± 6.7 percent were found when the urine samples were frozen during 40 days, 1 year, and 3 years, respectively.

2.3 Principle of liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is a column chromatography technique and the most useful tool in analytical chemistry. It is an analytical technique which liquid samples was pumped into a column filled with small solid particles (Stationary phase). Separation can occur in a columns, then used a mass spectrometer to ionize the separate substance (ion fragment) [19]. Figure 6 shows a diagram of the components of LC-MS.

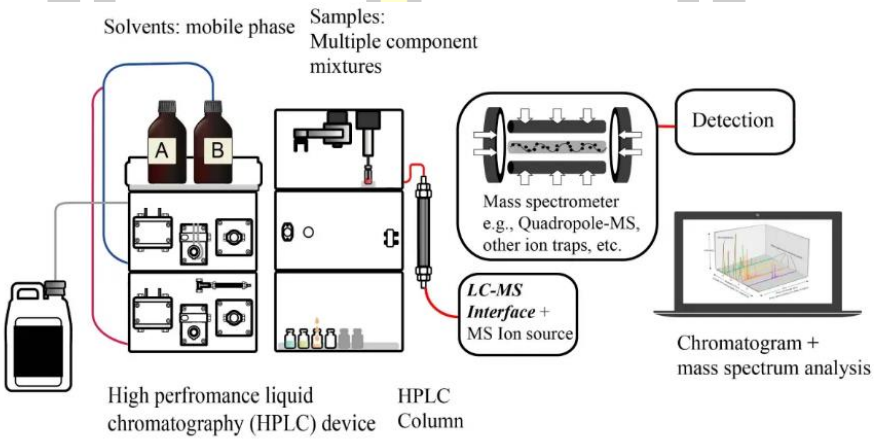

Figure 6 Liquid chromatography-mass spectrometry system [33].

LC-MS is a flexible technique. Various operating modes are used to customize analysis for certain sample types. Tunable parameters include ionization source, ionization polarity, dispersion and sensitivity. Electrospray (ESI) is the most common ionization source. This method is often used as the first type of analysis for samples in positive ESI mode or negative ESI mode. Atmospheric Pressure Chemical Ionization (APCI) is available in both positive and negative modes. This ionization technique is particularly useful for highly hydrophobic compounds such as petroleum products [34].

ESI is used in LC-MS. The positive ESI mode or negative ESI mode ionization is achieved by spraying the sample carried by the mobile phase into the ion source through a capillary needle while simultaneously applying high voltage. APCI is achieved with the aid of an electrical discharge applied to the sprayed molecules in the presence of nitrogen gas under atmospheric pressure.

Online extraction traps analytes were flowed through an extraction column in line with the LC system while flushing matrix to waste [35]. Then the mobile phase, and typically flow direction, are switched to elute analytes to either an analytical column for separation or directly to the MS detector. The online SPE system allows for automated sample clean-up to remove the sample matrix via a bidirectional trap column prior to being eluted for separation on the analytical column. The advantages of sample extraction by this technique will be more stable, use fewer samples, shorten working time and reduce the use of chemicals more than solid phase extraction (SPE) or liquid phase extraction (LLE). Figure 7 shows a diagram of the components of online SPE system.

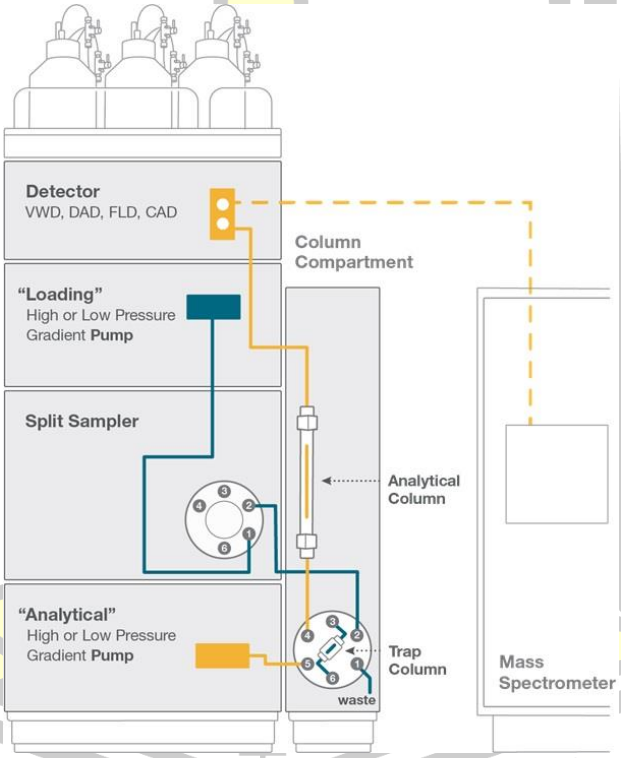

Figure 7 Online SPE system [36].

2.4 Extraction techniques

2.4.1) Hydrolysis

Hydrolysis is a chemical reaction that involves the breaking of covalent bonds in a molecule using water. This process results in the formation of two or more products. Hydrolysis is an important process in biological fluid. It is involved in various functions such as digestion, energy production, and the synthesis of biomolecules [37].

Yoshida et al., [37] discussed the discovery of a bacterium that is capable of degrading and assimilating polyethylene terephthalate (PET) a commonly used plastic. The bacterium secretes an enzyme that hydrolyzes the ester bonds in PET breaking it down into simpler compounds that the bacterium can use for energy and growth. This discovery has important implications for the development of new technologies for recycling and managing plastic waste.

2.4.2) Protein precipitation

Protein precipitation (PPT) is one of the most popular techniques used to prepare biological fluid (bio-fluid) samples for LC/MS/MS research analysis. By applying protein precipitation to bio-fluid samples, proteins can efficiently be removed from the matrix. PPT is the process for protein separated from any contaminants that may be mixed with it [38].

- Isoelectric precipitation is the process that changing the pH of the protein solution.

- Salting out precipitation is the process that increasing the salt concentration in soluble. The higher concentration of salt competes with the protein to bind with water. Then, the proteins bind themselves, folding and precipitating [1].

- Thermal precipitation, most proteins denature when the temperature exceeds 50 °C.

- Cationic precipitant requires adjusting the pH of the protein solution to a value higher than the isoelectric point of the protein.

- Anion precipitant requires adjusting the pH of the protein solution to a value below the isoelectric point of the protein.

- Organic solvent for precipitation was used organic solvents to decrease the dielectric constant. As a result, the solubility of the protein was decreased. There is an

interaction between the proteins together until the sediment falls. The problem with using organic solvents to precipitate proteins is heat generation.

2.4.3) QuEChERS

QuEChERS is a sample preparation technique based on the principle of solid sorbent extraction. It's a technique that can be done quickly. It is effective, easy to do, cheap and safe. This technique is divided into three parts. Extraction is phase separation and buffering. Sorbents for cleanup were used to remove unwanted coextracted. Tube was employed to hold the ingredients and samples during extraction and cleanup [39].

2.4.4) Captiva EMR

Agilent Captiva Enhanced Matrix Removal - Lipid (EMR - Lipid) sorbent was provided with highly selective and efficient removal of phospholipids and other lipids from biological fluids after protein precipitation. This product is also designed with a depth filtration mechanism. So, it provides efficient protein precipitate filtration without clogging and simplifies the workflow, ensuring protein and lipid removal [40]. The schematic representation of the captiva EMR was presented in Figure 8.

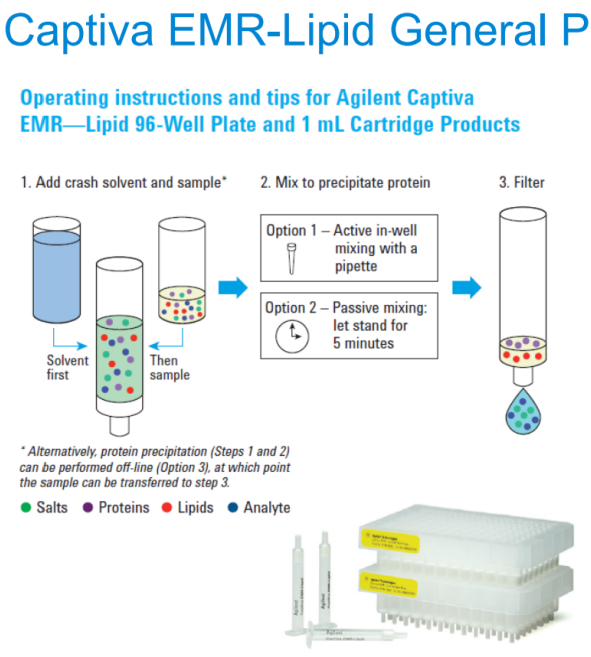

Figure 8 Schematic representation of the captiva EMR [40].

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used in this work were analytical grade and high performance liquid chromatography grade are listed in Table 2.

Table 2 List of chemicals used in this work.

No.	Chemicals	Formula	Grade/Purity	Company	Country
1.	11-Nor-Δ9-THC-COOH	$C_{21}H_{28}O_4$	99.71	Sigma-Aldrich	Germany
2.	11-Nor-Δ9-THC-COOH-d_3	$C_{21}H_{25}D_3O_4$	99.63	Sigma-Aldrich	Germany
3.	Acetonitrile	CH_3CN	HPLC	Merck	Germany
4.	Ammonium formate	NH_4HCO_2	99.0%	Sigma-Aldrich	Germany
5.	Formic acid	CH_2O_2	AR	Merck	Germany
6.	Methanol	CH_3OH	HPLC	Merck	Germany
7.	Sodium hydroxide	NaOH	AR	Merck	Germany
8.	Ultrapure Water (Type I)	-	-	Milli-Q, Millipore	Italy

AR : grade chemical means analytical reagent

HPLC : grade chemical means high performance liquid chromatography

3.2 Instrumentation

Liquid chromatography system consists of a Thermo scientific Vanquish™ UHPLC (USA), and an ISQ™ EC Single Quadrupole Mass Spectrometer was employed for this proposed. An Accucore™ C18 HPLC Column (2.1 × 150 mm, 2.6 µm) (Merck, Germany) was used as an analytical column and carried out at room temperature. A HyperSep Online SPE, retain PEP (2.1x20 mm) was used as an online SPE. The injection volume (10 µL) was transferred to Online-SPE with acetonitrile:water (50:50) at a flow rate of 0.3 mL/min. Chromeleon™ chromatography data system (CDS) software was used for data processing. The Δ9-tetrahydrocannabinol metabolite was separated using gradient elution with

acetonitrile:0.2 % formic acid in 10 mM ammoniumformate (90:10) as the mobile phase at a flow rate of 0.5 mL/min. A vortex mixer (50 Hz, model Genie 2, Scientific Industries, USA), Dry bath Incubator (50 Hz, Model MK200-2, ALL SHENG, China) and centrifuge (Model 5430, Eppendorf, USA) was used to mix the solution and accelerated the phase separation, respectively.

3.3 Experimental

3.3.1) Preparation of standard solution

3.3.1.1) Stock standard solution of 11-Nor-Δ9-THC-COOH 10,000 mg L^{-1} (WS10,000)

Stock 11-Nor-Δ9-THC-COOH 10,000 mg L^{-1} was prepared by pipette 100 µL of stock 11-Nor-Δ9-THC-COOH 1 mg mL^{-1} into 10 mL of volumetric flask and volume was adjusted volume to 10 mL with methanol. This solution was stored in a dark glass bottle at temperature -20°C. Working standard solutions was prepared daily from the stock solution by a stepwise dilution with negative urine in Table 3 and 4.

3.3.1.2) Stock standard solution of 11-Nor-Δ9-THC-COOH 1,000 mg L^{-1} (WS1,000)

Stock 11-Nor-Δ9-THC-COOH 1,000 mg L^{-1} was prepared by pipette 1,000 µL of stock 11-Nor-Δ9-THC-COOH 10,000 mg L^{-1} into 10 mL volumetric flask and volume was adjusted to 10 mL with methanol. The solution was kept in a dark glass bottle at temperature -20°C. Working standard solutions was prepared daily from the stock solution by a stepwise dilution with negative urine in Table 3 and 4.

Table 3 Standard solution for calibration curve (CS_1-CS_7).

Level	**WS1,000**	**WS10,000**	**Urine taken**	**Final Conc.**
	Taken volume (µL)	**Taken volume (µL)**	**Final volume (mL)**	**(ng mL^{-1})**
CS_1	10	-	10	1
CS_2	50	-	10	5
CS_3	100	-	10	10
CS_4	-	25	10	25
CS_5	-	50	10	50
CS_6	-	75	10	75
CS_7	-	100	10	100

Table 4 Standard solution for QC Sample.

QC Sample	Volume taken WS1,000 (µL)	Volume taken WS10,000 (µL)	Final volume in Urine (mL)	Final volume in water (mL)	Final Conc. (ng mL^{-1})
QCL	30	-	10	-	3
QCM	-	40	10	-	40
QCH	-	80	10	-	80
SS	-	40	-	10	40

QCL : quality control low

QCM : quality control medium

QCH : quality control high

SS : system suitability

3.3.1.3) Stock standard solution of 11-Nor-Δ9-THC-COOH-d_3 10,000 mg L^{-1}

Stock standard solution of 11-Nor-Δ9-THC-COOH-d_3 10,000 mg L^{-1} was prepared by pipette 100 µL of stock 11-Nor-Δ9-THC -COOH-d_3 1 mg mL^{-1} into 100 mL volumetric flask and the volume was adjusted to 100 mL with ultrapure water and was stored in a dark glass bottle at temperature 2-8 °C.

3.3.2) Preparation of 1 M ammonium formate

An ammonium formate 1 M was prepared by dissolving 12.6 g of ammonium formate in 200 mL of ultrapure water and this solution was stored in a dark glass bottle at temperature 2-8 °C.

3.3.3) Preparation of 0.2% formic acid in 10 mM ammonium formate

A formic acid 0.2% in 10 mM ammonium formate was prepared by pipette 1 mL of 1.0 M ammonium formate and formic acid 2 mL into 1,000 mL volumetric flask. Then, the final volume was adjusted to 1000 mL with ultrapure water. This solution was filtered through a nylon filter paper with a pore size of 0.2 micrometers.

3.3.4) Preparation of 10 M sodium hydroxide

A sodium hydroxide 10 M was prepared by dissolving 40.0 g of sodium hydroxide in 100 mL of ultrapure water and stored in a plastic bottle at temperature 2-8 °C.

3.4 The optimal conditions for sample preparation

3.4.1) Effect of sodium hydroxide

Sodium hydroxide is an important parameter for protein precipitate. The effect of pH was studied by varying the NaOH concentration at 5.0, 10.0, and 15.0 M and volume of NaOH in the range 10-30 µL was also investigated.

3.4.2) Effect of speed and time centrifugation

Centrifugation is another important parameter in procedure to achieve phase separation. In this method, it was studied in the range of 8,000-12,000 rpm and 5-15 min for speed and time centrifugation, respectively.

3.4.3) Effect of centrifugal temperature

In this method, it was studied at room temperature and 4 °C.

3.5 Optimal conditions for protein precipitation and removal of impurities

3.5.1) Sample preparation by hydrolysis

The sample preparation was carried out as follows. 11-Nor-Δ9-THC-COOH-d_3 100 µL was firstly added into a test tube (3 mL capacity). Then, 1 mL standard or sample solution was added. The solution was vortexed for 10 s and added 20 µL of 10 M sodium hydroxide. After that, the solution was mixed by using a vortex for 10 s and incubated at 60 ºC for 15 min. Then, the solution was left to cool at room temperature. Next, the mixing solution was pipetted into a microcentrifuge tube (2 mL capacity) for centrifugation at 10,000 rpm for 10 min to complete phase separation of precipitate from the aqueous solution. Approximately 600 µL of the supernatant was aspirated into a 1.8 mL vial for analysis by LC-MS. The diagram for protein precipitation was illustrated in Figure 9.

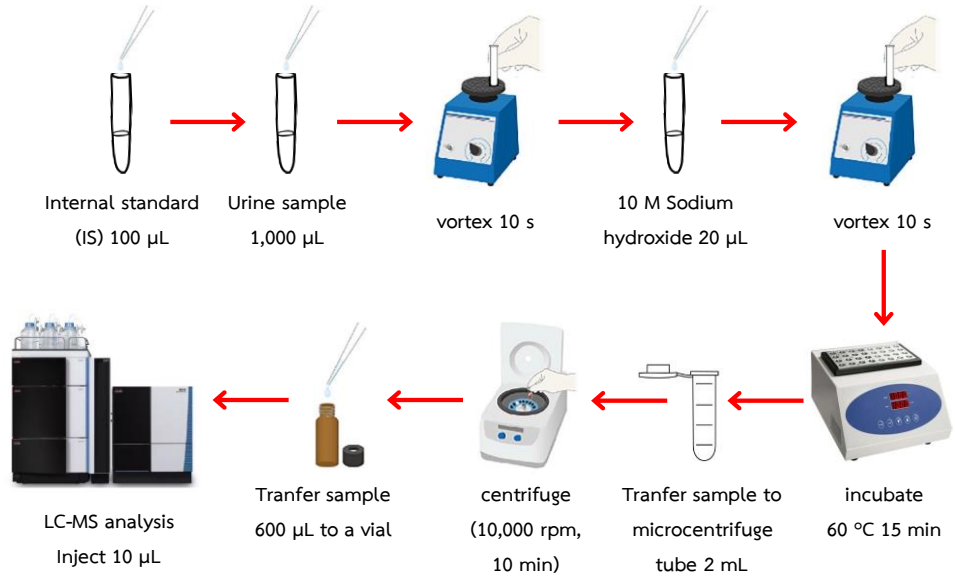

Figure 9 Simplified schematic view of the procedure for preparing urine samples by hydrolysis.

3.5.2) Sample preparation by lipid removal

Sample preparation for lipid removal was carried out as follows. 11-Nor-Δ9-THC-COOH-d_3 internal standard at 100 µL was firstly added into a test tube (3 mL capacity). Then, 1 mL standard or sample solution was added and vortexed for 10 s before adding 20 µL of 10 M sodium hydroxide. After that, the solution was mixed by using a vortex for 10 s. Sample solution was filtered by captive EMR to ensure completely remove protein and lipid. Sample was incubated at 60 ºC for 15 min and then left to cool at room temperature. The mixing solution was transferred into a microcentrifuge tube (2 mL capacity) and centrifuged at 10,000 rpm for 10 min to complete the isolation of precipitate from the aqueous solution. Approximately 600 µL of the supernatant was aspirated into a 1.8 mL vial for analysis by LC-MS. Diagram for lipid removal was showed in Figure 10.

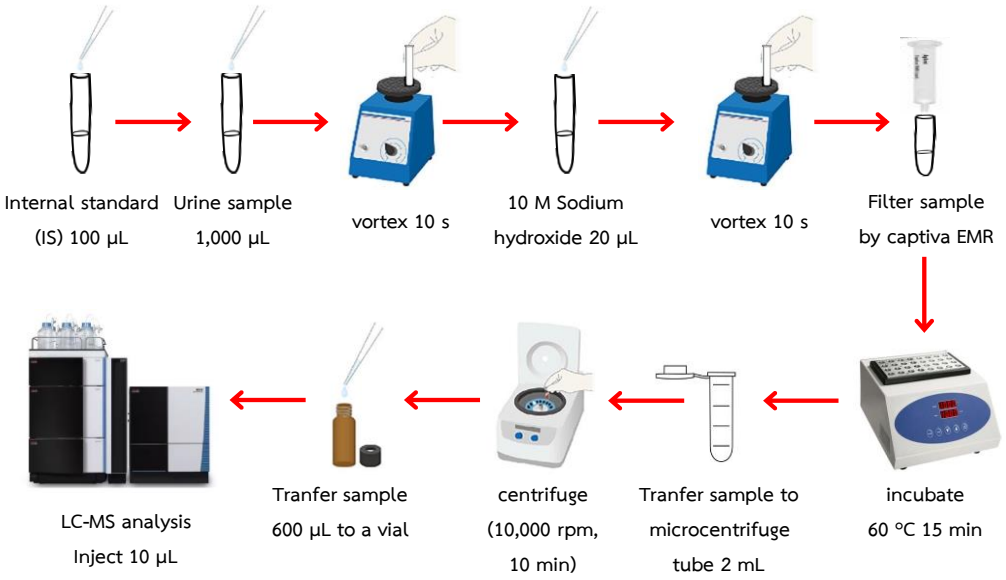

Figure 10 Simplified schematic view of the procedure for preparing urine samples by lipid removal.

3.5.3) Sample preparation by QuEChERS extraction

Sample preparation by QuEChERS was carried out as follows. Internal standard 11-Nor-Δ9-THC-COOH-d_3 100 µL was firstly added into a test tube (3 mL capacity). Then, 1 mL standard or sample solution was added and vortexed for 10 s. Next, 20 µL of 10 M sodium hydroxide was added. The solution was mixed by using a vortex for 10 s and sample was extracted by QuEChERS (QuEChERS extraction kit, EN 15662 method, Kit contents, 4 g $MgSO_4$, 1 g NaCl, 1 g NaCitrate, 0.5 g disodium citrate sesquihydrate) at 60 ºC for 15 min. After that, the sample solution was left to cool at room temperature before transferred into a microcentrifuge tube (2 mL capacity). The sample solution was centrifuged at 10,000 rpm for 10 min to complete phase separations. Approximately 600 µL of the supernatant was aspirated into a 1.8 mL vial for analysis by LC-MS. Schematic view of the procedure for preparing urine samples by QuEChERS method was presented in Figure 11.

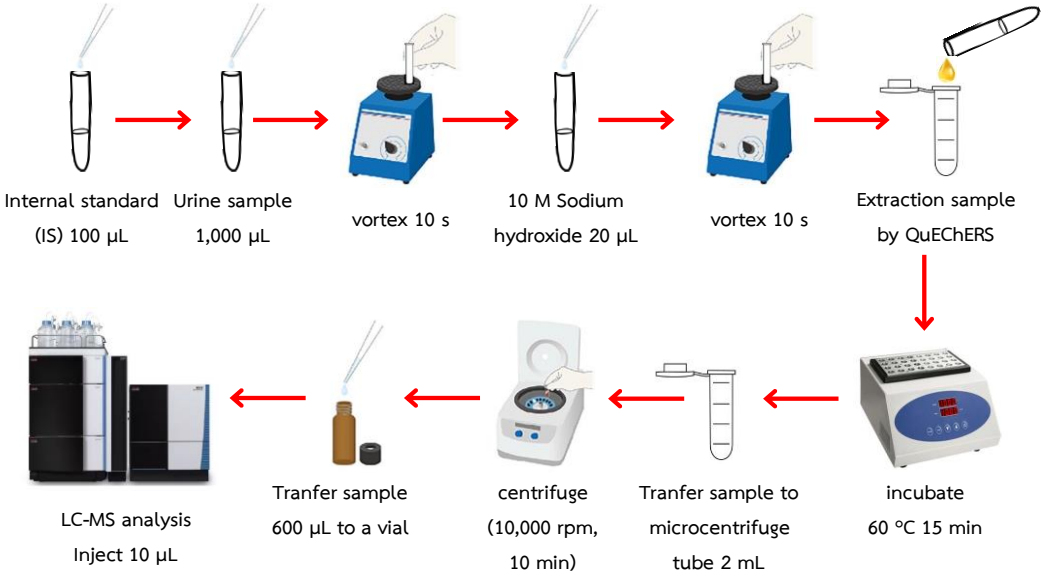

Figure 11 Simplified schematic view of the procedure for preparing urine samples by QuEChERS extraction.

3.6 The appropriate conditions for analysis

3.6.1) Effect of incubation time and temperature

Another parameter needing optimization was the effect of incubation time and temperature. Temperature is an important parameter and required for digestion sample in dry bath incubator. In order to optimize the effect of incubation time on dry bath incubator, it was optimized between 5 to 25 min. Incubation temperature was studied in the range 40-70 ºC.

3.6.2) Effect of sample injection volume

The impact of sample injection volumes of 1, 5, 10, 15, 20, and 25 µL was investigated to enhance the concentration using online-SPE before analysis with LC-MS.

3.6.3) Effect of duration times in the online-SPE process

The appropriate loading times was tested at 0.5, 1, 2, 3, and 4 min and the elution times was studied at 0.5, 1, 2, 3, and 4 min.

3.7 Method validation

The method was validated according to the U.S. Department of Health and Human Services, Food and Drug Administration and the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines [41, 42]. The validation parameters performed were specificity, carryover, limit of detection (LOD), limit of quantification (LOQ), linearity and range, accuracy, intra-day and inter-day precision, recovery, matrix effect, dilution integrity and stability. The standard solution was prepared and working solution was diluted in negative urine before injected into LC-MS under the optimum conditions.

3.7.1) Specificity

Specificity describes the ability of the bioanalytical method to produce a signal only for the analyte of interest and not for other interfering components. To assess endogenous matrix interferences, five blank samples were examined. The blank sample was fortified with additional common drugs of abuse, metabolites, and therapeutic-drugs. The blank sample was free from any interference, as evidenced by the retention times (t_R) of both the analyte and internal standard. The response of the analyte's peak at t_R was less than 20% of the response of the lower limit of quantitation, while the response of the internal standard utilized in the investigation was less than 5%.

3.7.2) Carryover

Carryover was evaluated by inject the extract blank matrix sample after the injection of high concentration of standard solution at 100 ng mL^{-1}. The signal of target analyte was not observed in the blank sample. Therefore, the method was free from carryover.

3.7.3) Linearity and range

A calibration curve for each analyte was constructed by plotting between the peak areas versus the concentration of standard solution at seven different concentrations of 1, 5, 10, 25, 50, 75 and 100 ng mL^{-1}. The linearity range was evaluated by the calibration curve ($y = mx + c$) and the correlation coefficient (R^2) was greater than 0.95.

3.7.4) Accuracy, precision and recovery

Method precision and accuracy was determined according to the SWGTOX guidelines, at three concentration levels (3, 40 and 80 ng mL^{-1}). For the intra-day precision, a total of three sample replicates was extract and analyzed for each of the concentrations in a single day. And the inter-day precision, the same procedure was repeated along three days. The intra-day and inter-day precisions was determined by calculating within ± 20% coefficient of variation (%CV), while the accuracy was calculated as the relative difference between the mean concentration values resulting from the analysis of sample replicates and their nominal concentrations. According to the SWGTOX guidelines, accuracy values (%Bias) within ± 20% was considered acceptable. The recovery test was performed by analysis ten blank urine samples, at three concentration levels (3, 40 and 80 ng mL^{-1}) which extracted in accordance with the previously described procedure. Recovery of the analyte was not obtained 100%, but the extent of recovery of an analyte and internal standard was consistent, precise, and reproducible.

3.7.5) Limit of detection and limit of quantitation

Sensitivity of the method was evaluated by limit of detection (LOD) calculated as three times the signal-to-noise ratio (3:1), and limit of quantitation (LOQ) was calculated as ten times the signal-to-noise ratio (10:1).

3.7.6) Matrix effect

The matrix effect was determined from accuracy and precision of each ten lots of samples spiked at two concentration levels (3 and 80 ng mL^{-1}). The concentration of the analyte for each source was calculated and compared to the calibration curve. Coefficient of variation (%CV) and bias (%) at ± 20% were considered acceptable.

3.7.7) Dilution integrity

Dilution integrity was studied to confirm the ability of dilution samples originally above ULOQ (100 ng mL^{-1}). Sample dilution was determined by analyzing replicates ($n \geq 5$) of each dilution factor (e.g., 1:2, 1:5, 1:10, 1:20) against a freshly prepared calibration curve. Coefficient of variation (%CV) and bias (%) within ± 15% were considered acceptable.

3.7.8) Stability

Stability was evaluated by analyzing the extract sample at two concentration levels of 3 and 80 ng mL^{-1} which was stored in the autosampler at 8, 24 h and the standard was re-analyzed after stored in 2-8 °C after 72 h. Stability was considered acceptable by coefficient of variation (%CV) and bias (%) within ± 15%.

3.8 Data analysis

3.8.1 The average result (mean) was calculated by summing the individual result and dividing by the number (n) of individual values:

$$\bar{X} = \frac{x_1+x_2+x_3+...}{n}$$

$\bar{X}$ = average value
x = measured value
n = count of measured

3.8.2 The standard deviation was measured. It was calculated as follows:

$$SD = \sqrt{\frac{(x_1-\bar{x})^2+(x_2-\bar{x})^2+(x_3-\bar{x})^2+\ldots}{n-1}}$$

SD = standard deviation
$\bar{X}$ = average value
x = measured value
n = count of measured

3.8.3 The percentage bias (%Bias) was calculated by concentration of measure concentration divided nominal concentration using the equation:

$$\%Bias = \frac{\text{Measured concentration-Nominal Concentration}}{\text{Nominal Concentration}} \times 100$$

"%Bias" refers to the percentage difference or deviation between an observed or measured value and a true or expected value. It serves as a measure of systematic error within a measurement or estimation process.

3.8.4 The percentage coefficient of variation (%CV) was calculated from the standard deviation and mean using the equation:

$$\%CV = \frac{SD \times 100}{\bar{x}}$$

%CV = coefficient of variation
SD = standard deviation
$\bar{X}$ = average value

3.8.5 The percentage recovery (%Recovery) was calculated by concentration of sample and spiked sample using the equation:

$$\%Recovery = \frac{(\text{Measured concentration of spiked sample})}{\text{Spiked concentration}} \times 100$$

"%Recovery" refers to the percentage of a substance or quantity that is recovered or obtained from a process, experiment, or operation compared to the expected or theoretical amount.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 The optimal conditions for sample preparation

4.1.1) Effect of sodium hydroxide

The employing of sodium hydroxide in alkaline hydrolysis proved to be both time-efficient and repeatable, while also being cost-effective [43]. In this study, the impact of sodium hydroxide solutions on sample preparation was performed at concentrations of 5, 10, and 15 M in volumes of 10, 20, and 30 µL. Solution pH was measured before, after adding NaOH, and after incubation. It was observed that the pH of the sample increased from 7 to 14 upon NaOH addition. The experiment revealed that the utilization of sodium hydroxide with a concentration of 10 M at a volume of 20 µL to adjust the pH to 14 resulted in the greatest peak area, as depicted in Figure 12-13. Therefore, sodium hydroxide at 10 M and 20 µL was selected to further experiment.

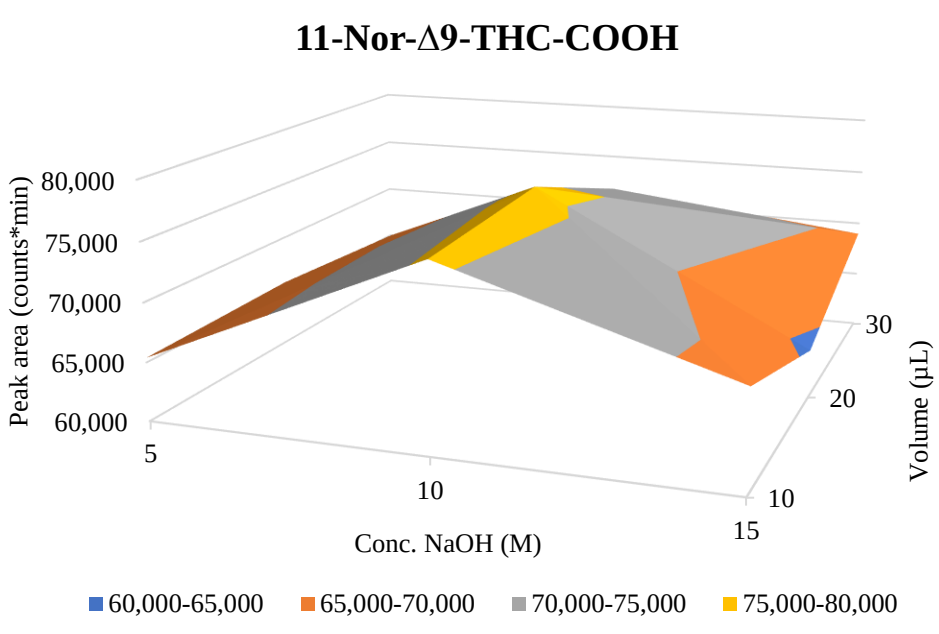

Figure 12 Peak area of 11-Nor-Δ9-THC-COOH in different concentrations and volumes.

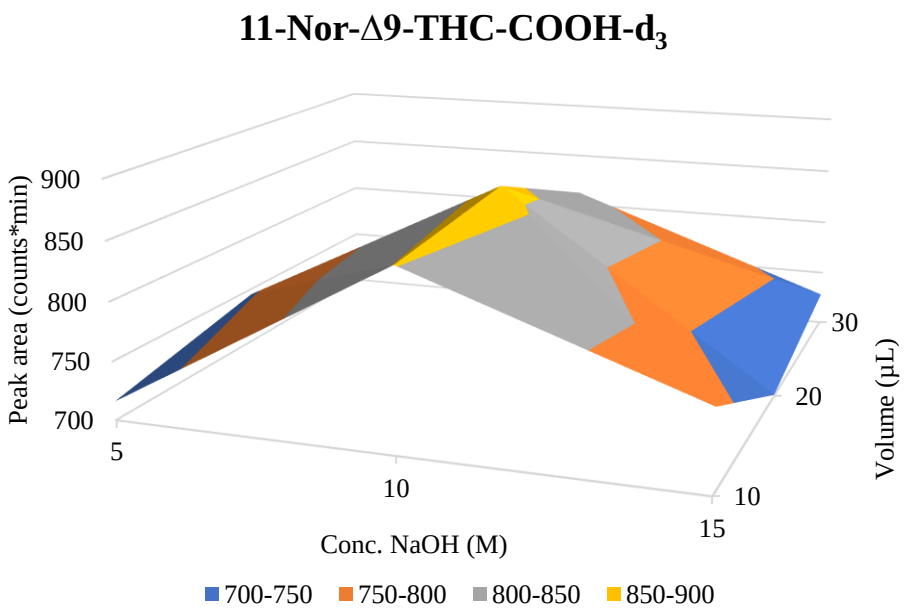

Figure 13 Peak area of 11-Nor-Δ9-THC-COOH-d_3 in different concentrations and volumes.

4.1.2) Effect of speed and time centrifugation

The effect of speed and time on the centrifuging sample at 8,000–12,000 rpm for 5, 10, and 15 min was studied. It was found that centrifugation at 10,000 rpm for 10 minutes provided the highest peak area, as shown in Figures 14–15.

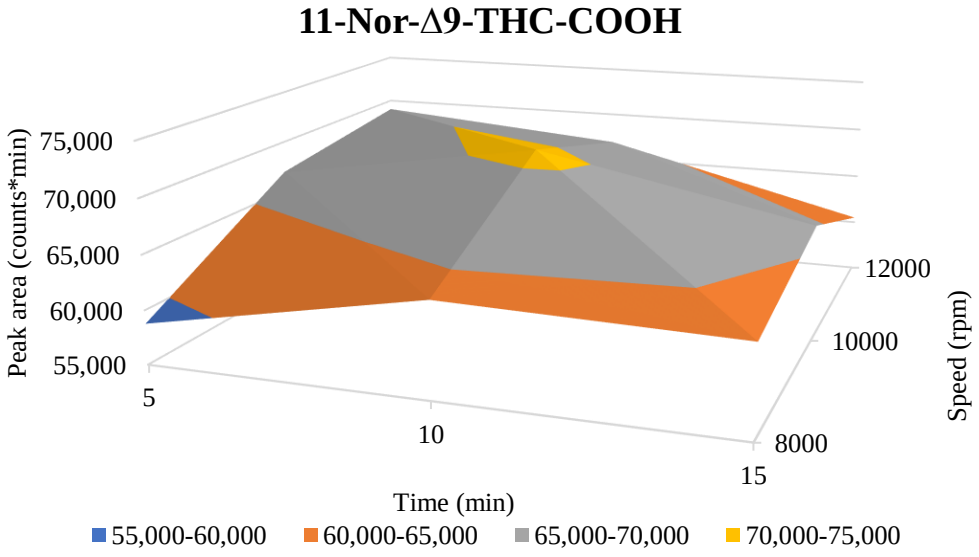

Figure 14 Peak area of 11-Nor-Δ9-THC-COOH in different speed and time centrifugation.

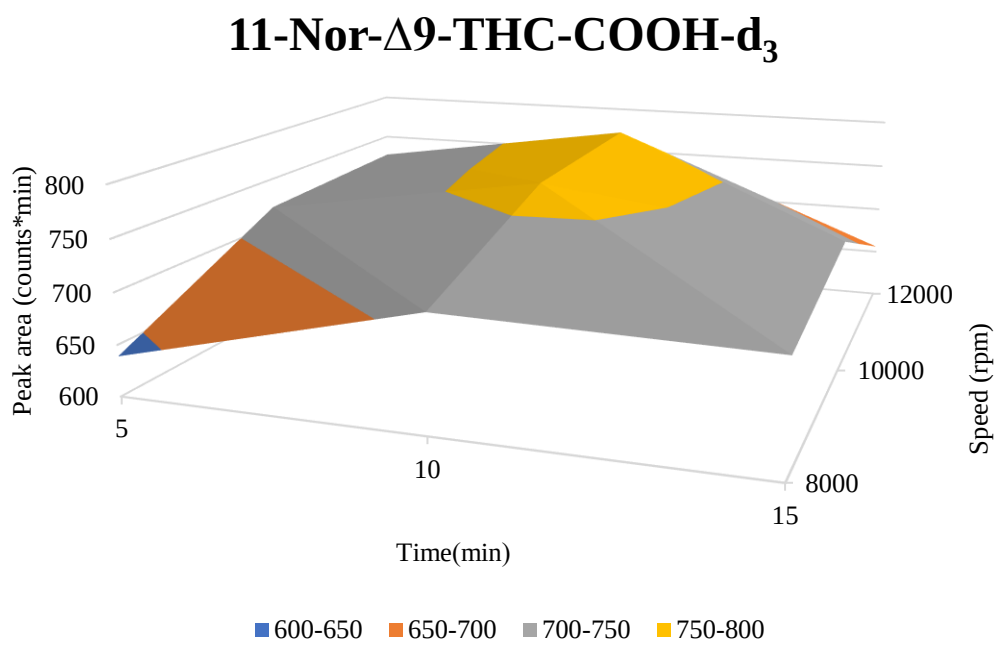

Figure 15 Peak area of 11-Nor-Δ9-THC-COOH-d_3 in different speed and time centrifugation.

4.1.3) Effect of centrifugal temperature

The impact of incubation temperature was examined at both ambient temperature and 4 ℃. It was found that centrifugation at 10,000 rpm for 10 minutes at room temperature and 4 ℃ produced similar experimental results. Consequently, room temperature was chosen for further experiments, as shown in Figure 16.

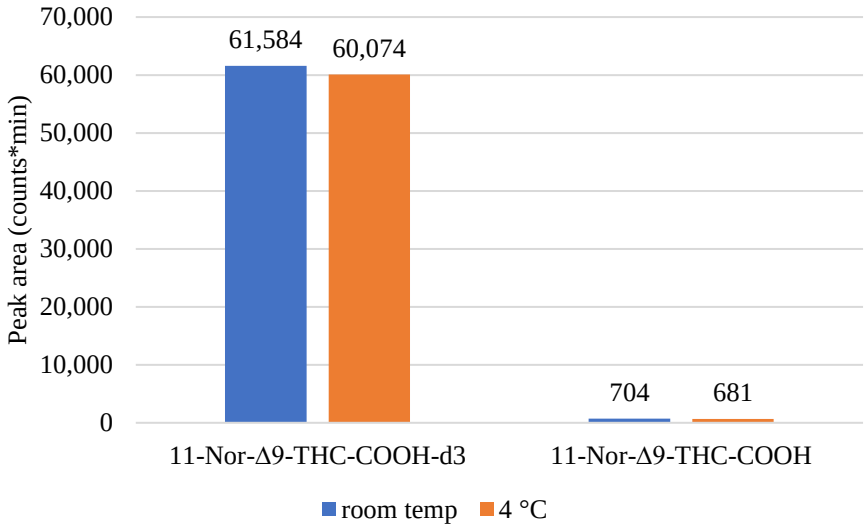

Figure 16 Peak area of 11-Nor-Δ9-THC-COOH and 11-Nor-Δ9-THC-COOH-d_3 after centrifugation at 10,000 rpm for 10 min at room temperature and 4 °C.

4.2 Optimal conditions for protein precipitation and removal of impurities

In this study, sample preparation via hydrolysis provided optimal peak areas without interference at the positions of 11-Nor-Δ9-THC-COOH and 11-Nor-Δ9-THC-COOH-d_3. The use of Captiva EMR was beneficial in removing lipids from the samples. This is particularly significant because THC-COOH is bound to lipid tissues, which could potentially be entrapped within the Captiva EMR. Additionally, applying QuEChERS for protein precipitation resulted in lower experimental results compared with hydrolysis. Therefore, hydrolysis is sufficient for protein precipitation in urine samples. Employing a dry bath incubator and an ultrasonic bath for sample preparation produced comparable peak areas.

After investigating suitable sample preparation conditions according to all three methods, it is evident that the hydrolysis sample preparation method with sodium hydroxide under a dry bath incubator produced superior peak areas, making it the most suitable approach for sample preparation, as shown in Figure 17–18.

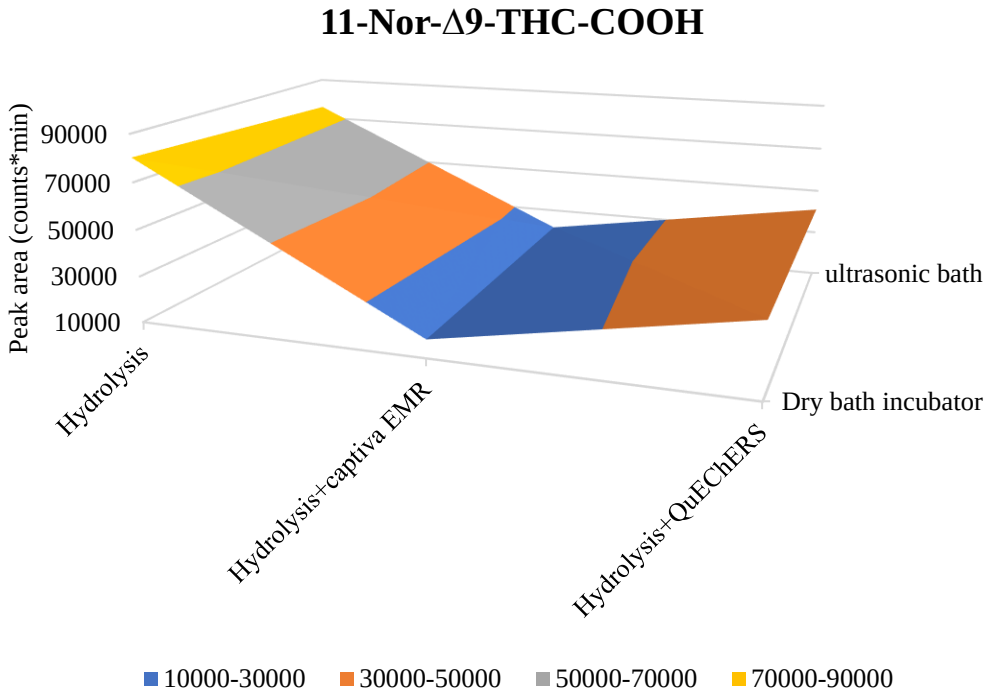

Figure 17 Peak area during sample preparation of 11-Nor-Δ9-THC-COOH by hydrolysis, lipid removal (hydrolysis + captiva EMR) and QuEChERS extraction (hydrolysis + salting out).

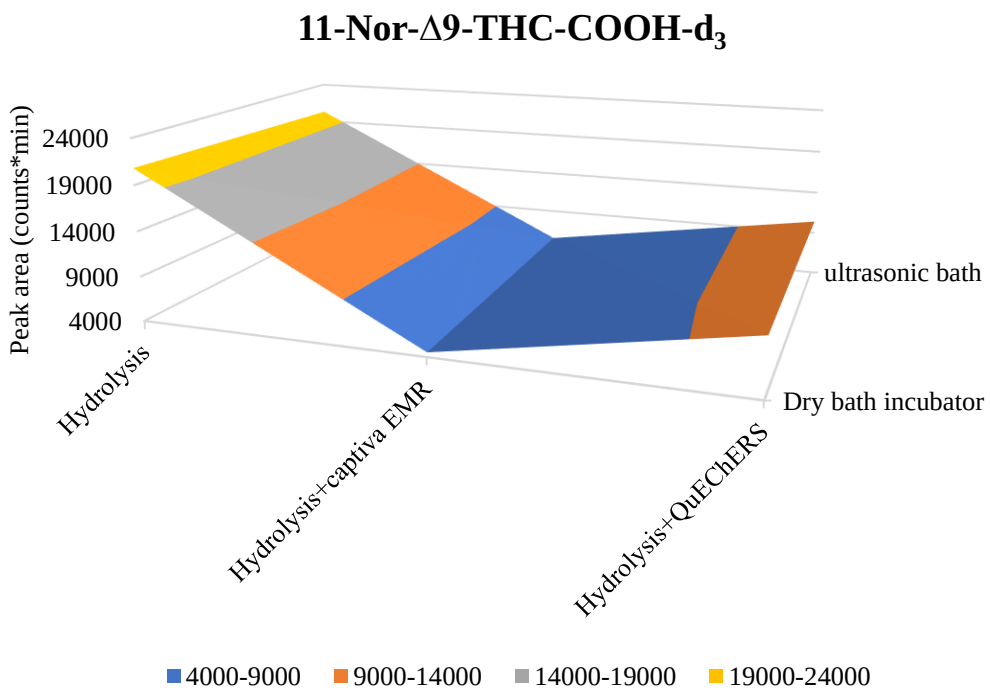

Figure 18 Peak area during sample preparation of 11-Nor-Δ9-THC-COOH-d_3 by hydrolysis, lipid removal (hydrolysis + captiva EMR) and QuEChERS extraction (hydrolysis + salting out).

4.3 The appropriate conditions for analysis

4.3.1) Effect of incubation time and temperature

The effects of incubation time and temperature were investigated at 5, 10, 15, 20, and 25 min and at 40, 50, 60, and 70 °C, respectively. It was found that an incubation time of 10 min at 40 °C provided the greatest peak area, and 20 min at 40 °C gave the lower peak area, as shown in Table 8 and Figures 19–20.

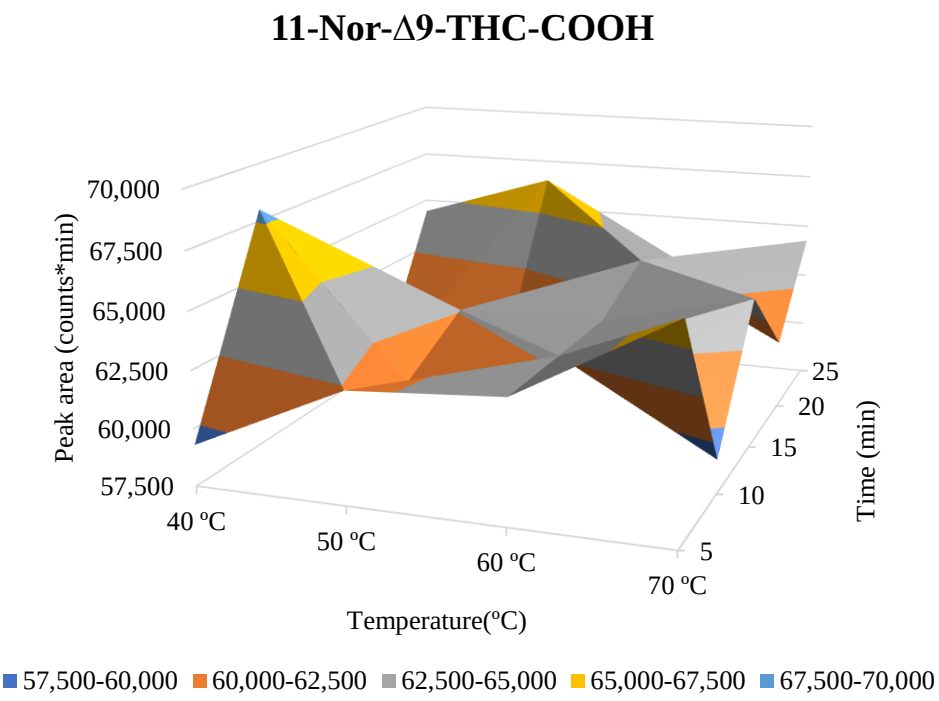

Figure 19 Peak area of 11-Nor-Δ9-THC-COOH in different incubation time and temperature.

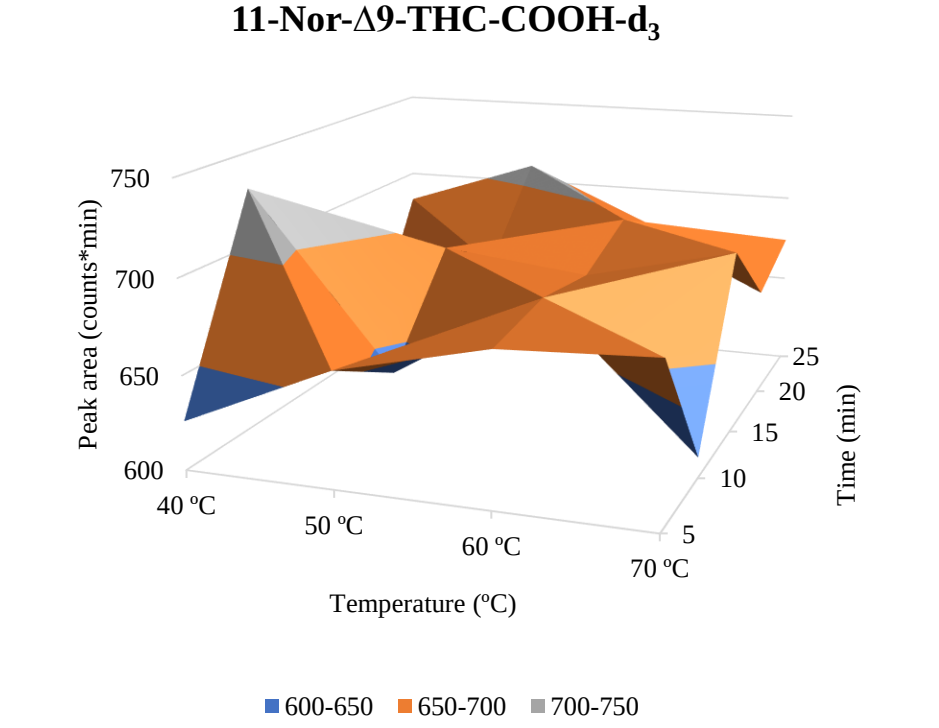

Figure 20 Peak area of 11-Nor-Δ9-THC-COOH-d_3 in different incubation time and temperature.

4.3.2) Effect of sample injection volume

The effects of sample injection volumes of 1, 5, 10, 15, 20, and 25 µL were studied. When a very large volume of sample is injected into the HPLC column, the peaks begin to front more (peak symmetry factor < 1), and the retention time may decrease, resulting in a decline in column efficiency and separation resolution [44, 45]. It was found that the sample injection volume of 10 µL gave the suitability peak area of 11-Nor-Δ9-THC-COOH and IS (11-Nor-Δ9-THC-COOH-d_3) as presented in Figure 21.

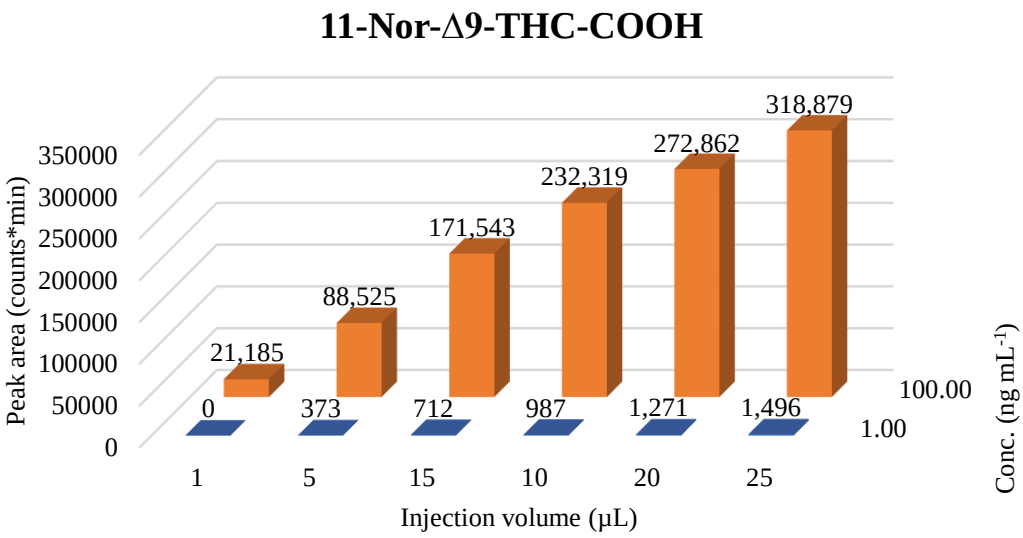

Figure 21 Peak area in different sample injection volume.

4.3.3) Effect of duration time in the online-SPE process

The optimum time for loading the sample was studied at 30 s, 1, 2, 3, and 4 minutes. It was determined that the duration of sample loading, which was set at 30 s, was insufficient, resulting in the sample not being captured by the online-SPE system. The duration of the sample load section varied between 2 minutes and longer, resulting in the removal of certain samples from the online-SPE process. Therefore, the sample loading time of 1 minute was optimum, as shown in Figure 22.

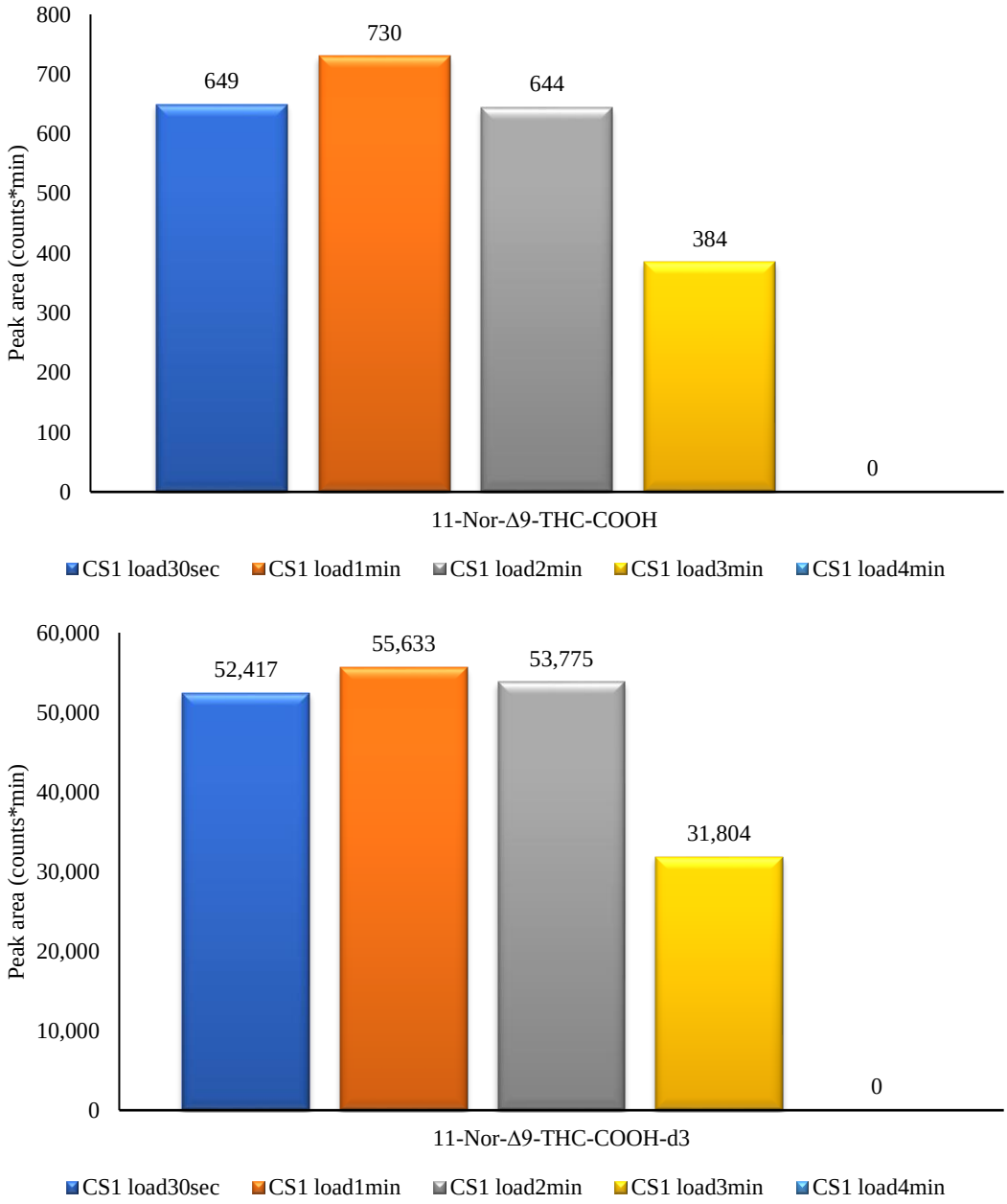

Figure 22 Peak area from loading samples at 0.5, 1, 2, 3, and 4 min.

The appropriate time for the elution analyte was studied at 30 s, 1, 2, 3, and 4 minutes. The elution durations of 30 s and 1 min were insufficient to elute the analyte from online SPE. Therefore, the sample is not completely eluted, resulting in low sensitivity. The sample was diluted with the analyte for more than 2 minutes. As a result, one minute is a suitable sample elution time, as shown in Figure 23.

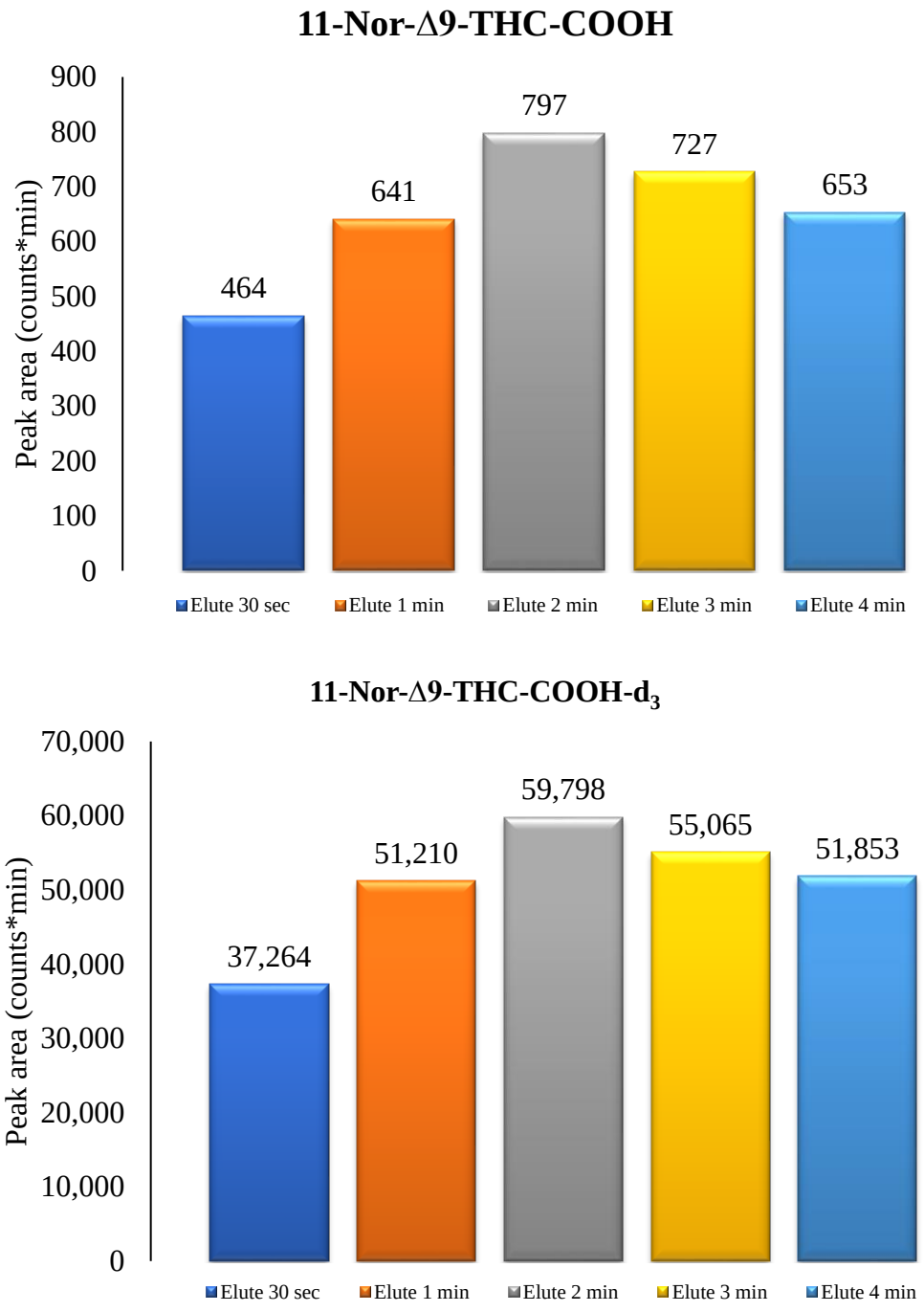

Figure 23 Peak area from elution samples at 0.5, 1, 2, 3, and 4 min.

4.4 Method validation

The analytical performance of the developed method was validated according to the criteria described in Chapter III: 3.7 Method Validation.

4.4.1) Specificity

An interfering peak was not observed in at least ten different batches of blank urine at the retention times of analyses and internal standards. Therefore, this method was specified for the determination of 11-Nor-Δ9-THC-COOH. Chromatograms are shown in Figures 24–25.

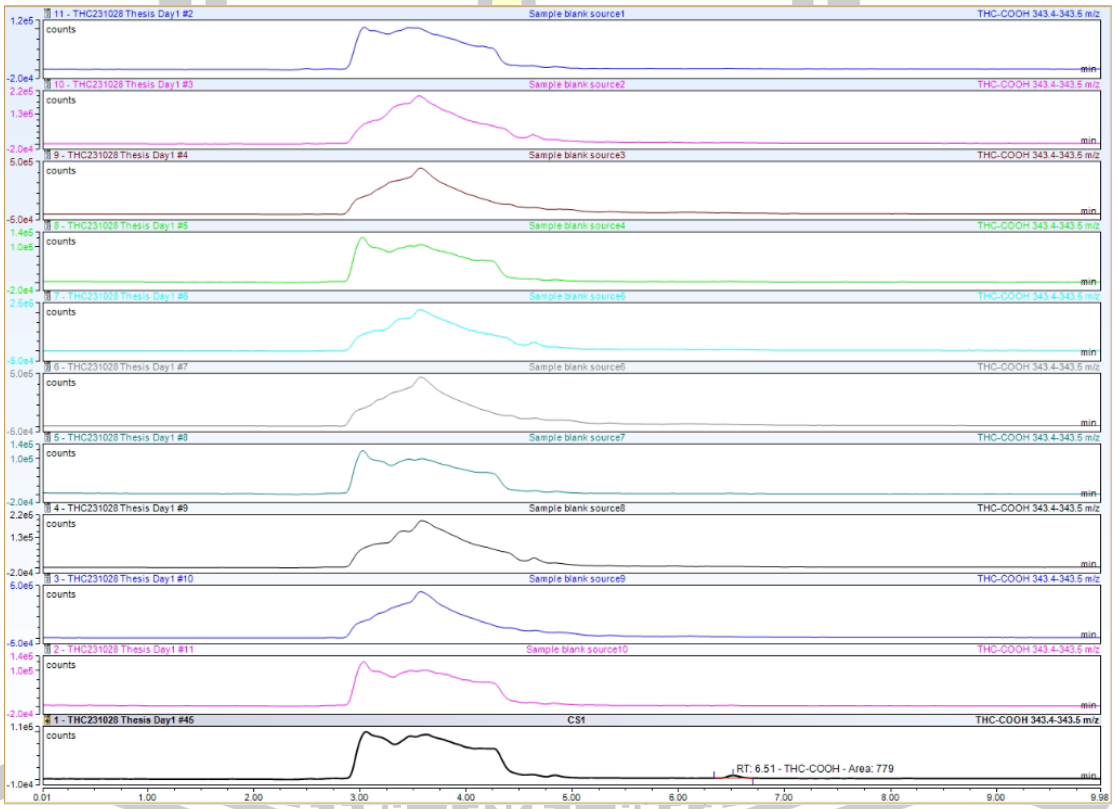

Figure 24 Chromatograms of 11-Nor-Δ9-THC-COOH with ten blank urine sources.

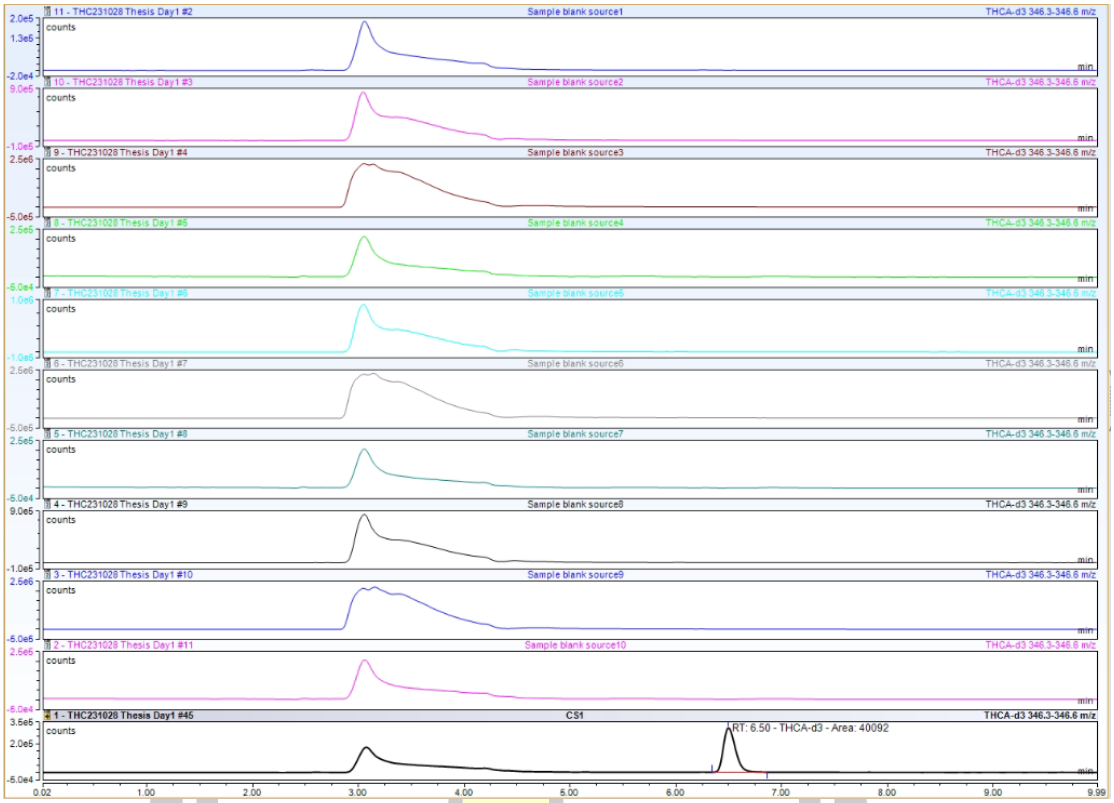

Figure 25 Chromatograms of 11-Nor-Δ9-THC-COOH-d_3 with ten blank urine sources.

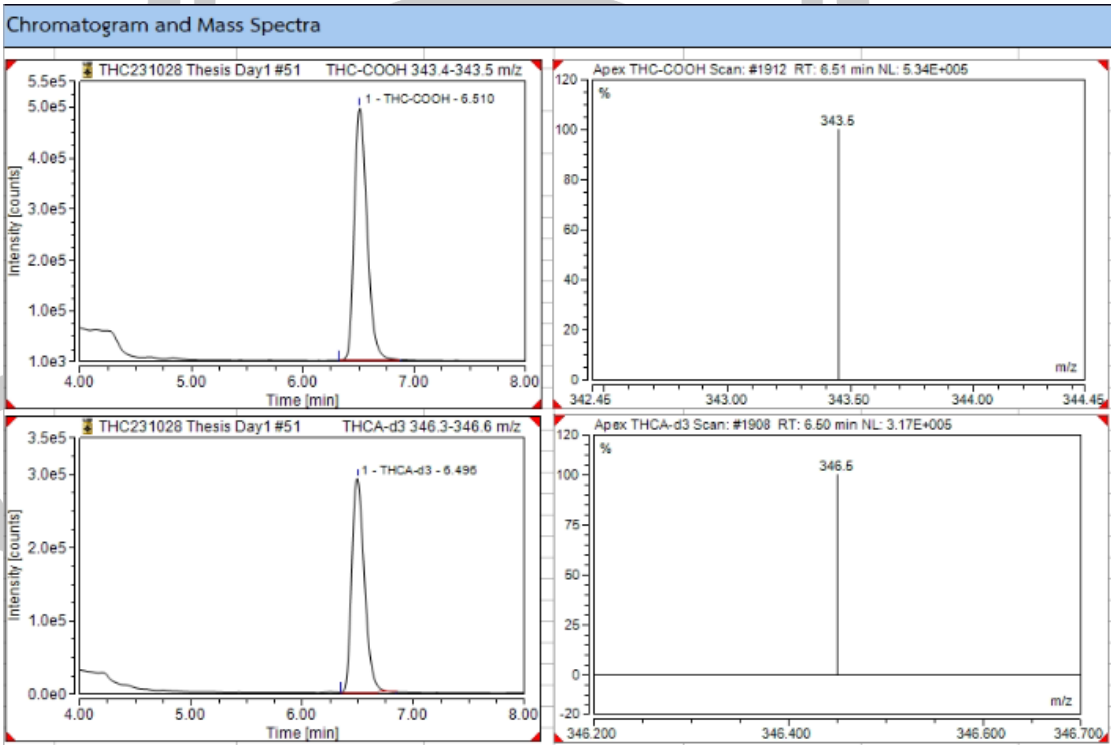

Figure 26 Chromatogram and mass spectra of 11-Nor-Δ9-THC-COOH and 11-Nor-Δ9-THC-COOH-d_3

4.4.2) Carryover

Carryover was investigated by injecting a sample blank before and after the ULOQ. It was discovered that there was no observable carryover in the system, and it had no effect on determining the sample at high concentration, as shown in Table 5.

Table 5 Carryover values of 11-Nor-Δ9-THC-COOH.

Name	Normal conc. [ng mL^{-1}]	Peak Area	Peak Area IS	Peak Area Ratio	Cal. conc. [ng mL^{-1}]	Bias[41] [%]
In_blank2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
sample blank S1-1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ULOQ S1-1	100.000	58897.350	34903.712	1.687	94.457	-5.54
ULOQ S1-2	100.000	61723.042	36756.474	1.679	93.999	-6.00
ULOQ S1-3	100.000	62957.548	37040.815	1.700	95.143	-4.86
sample blank S1-2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LLOQ S1	1.000	941.311	47236.370	0.020	1.090	8.952
sample blank S1-3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
In_blank3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
sample blank S2-1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ULOQ S2-1	100.000	61215.474	36207.241	1.691	94.640	-5.360
ULOQ S2-2	100.000	67234.143	39546.466	1.700	95.168	-4.83
ULOQ S2-3	100.000	82277.143	48110.641	1.710	95.730	-4.27
sample blank S2-2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LLOQ S2	1.000	814.041	42445.097	0.019	1.048	4.759
sample blank S2-3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
In_blank4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
sample blank S3-1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ULOQ S3-1	100.000	68060.258	39702.999	1.714	95.958	-4.042
ULOQ S3-2	100.000	79088.622	46372.314	1.706	95.469	-4.531
ULOQ S3-3	100.000	70132.499	41263.988	1.700	95.139	-4.86
sample blank S3-2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LLOQ S3	1.000	808.604	41160.086	0.020	1.074	7.37
sample blank S3-3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
In_blank5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. : not available

4.4.3) Linearity and range

The linearity of the 11-Nor-Δ9-THC-COOH was found in the range of 1.00–100.00 ng mL^{-1}. The LLOQ concentration of 11-Nor-Δ9-THC-COOH was 1.00 ng mL^{-1}. The correlation coefficient (r) was greater than 0.999. The back-calculated value of each concentration level was accepted within the criteria as shown in Tables 6-7 and Figure 27.

The calibration curve was repeated seven times per level to test the calibration model. It was found that the correlation coefficient (r) was greater than 0.999, as shown in Table 8 and Figure 28.

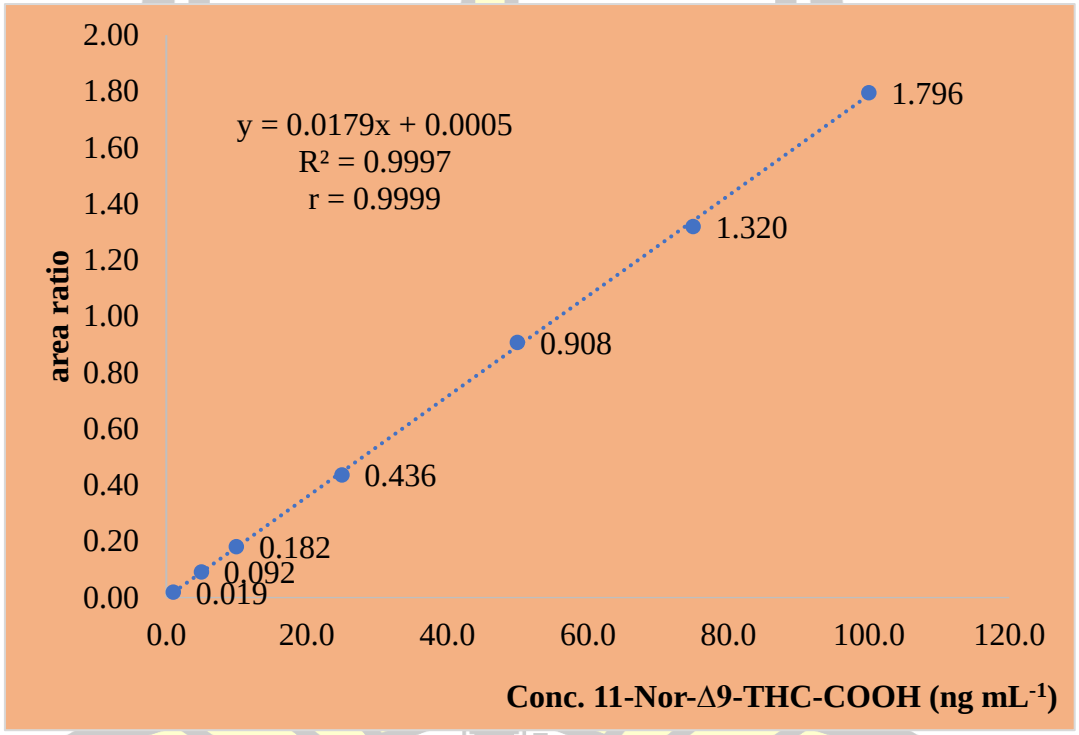

Figure 27 Calibration curves of 11-Nor-Δ9-THC-COOH.

Table 6 Calibration standard data.

Calibration curves		11-Nor-Δ9-THC-COOH concentration (ng mL^{-1})						
		1.000	**5.000**	**10.000**	**25.000**	**50.000**	**75.000**	**100.000**
Run 1	**Cal.conc.**	1.062	5.109	10.155	24.414	50.821	73.896	100.542
	Accuracy(%Bias)	6.19	2.19	1.55	-2.34	1.64	-1.47	0.54
Run 2	**Cal.conc.**	0.971	4.783	9.903	25.249	49.816	75.912	99.367
	Accuracy(%Bias)	-2.93	-4.35	-0.97	1.00	-0.37	1.22	-0.63
Run 3	**Cal.conc.**	0.928	4.917	9.736	24.859	50.281	76.410	98.868
	Accuracy(%Bias)	-7.19	-1.66	-2.64	-0.56	0.56	1.88	-1.13
mean		0.987	4.936	9.932	24.841	50.306	75.406	99.592
SD		0.07	0.16	0.21	0.42	0.50	1.33	0.86
%Bias		-1.31	-1.27	-0.68	-0.64	0.61	0.54	-0.41
%CV		6.93	3.33	2.13	1.68	1.00	1.77	0.86

Table 7 Calibration curve parameters.

Calibration curve	Regression parameters			
	Slope	**Intercept**	**Correlation coefficient (r)**	**Coefficient of determination (R^2)**
Day 1	0.0179	0.0005	0.9999	0.9997
Day 2	0.0216	0.0005	0.9999	0.9998
Day 3	0.0212	0.0029	0.9998	0.9996

Table 8 Calibration curve of 11-Nor-Δ9-THC-COOH with 7 replicates (n = 7).

level	Concentration	mean conc. (ng mL^{-1})	SD	%CV	%Bias
1	1.00	0.990	0.05	4.63	-1.02
2	5.00	4.614	0.05	1.17	-7.72
3	10.00	9.793	0.08	0.81	-2.07
4	25.00	22.737	0.14	0.60	-9.05
5	50.00	48.692	0.35	0.72	-2.62
6	75.00	75.408	0.14	0.18	0.54
7	100.00	99.824	0.60	0.60	-0.18

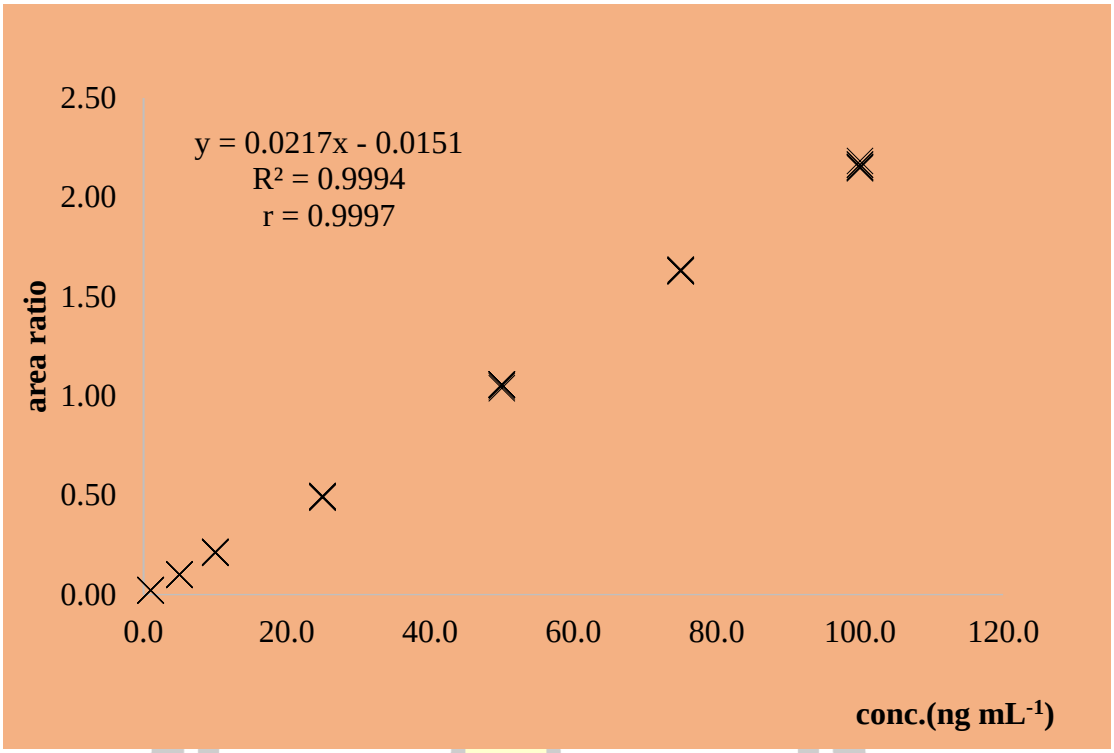

Figure 28 Calibration model of 11-Nor-Δ9-THC-COOH

4.4.4) Accuracy, precision and recovery

The accuracy is demonstrated by the %Bias values. It was found that %Bias was not exceeded by ±3% across all concentration levels. Precision is evaluated by the coefficient of variation (CV) values. %CV was not exceeded by ±4% across all concentration levels. These values fell within the acceptance criteria, indicating that the analytical method possesses high accuracy and good reliability, as depicted in Tables 9–10. One-way ANOVA of QC sample found that calculated F value (F_{cal}) was lower than the critical F value (F_{crit}) and P-value was greater than 0.05. These results indicate that there is no significant difference, as shown in Table 11.

The recovery values range from 99.16% to 101.60%, falling within the acceptance criteria. This demonstrates that the analysis method was accurate and reliable, as depicted in Table 10.

Table 9 Intra-day precision and accuracy of QC sample

Concentration level	Nominal conc. ($ng\ mL^{-1}$)	Back-calculated concentration ($ng\ mL^{-1}$)		
		Run 1	**Run 2**	**Run 3**
LLOQ	1.00	1.0172	1.0020	1.0244
		1.0417	1.0934	1.0172
		1.0578	1.0879	1.0172
		0.9610	1.0173	1.0156
		1.0071	1.0650	1.0132
		1.0034	0.9575	1.0232
		1.0844	1.0352	1.0098
	Mean	**1.0246**	**1.0369**	**1.0172**
	SD	**0.0405**	**0.0491**	**0.0052**
	%CV	**3.95**	**4.74**	**0.51**
	%Bias	**2.46**	**3.69**	**1.72**
QCL	3.00	3.0777	3.0492	3.0167
		3.0877	3.0698	3.0934
		3.0775	3.0481	3.0789
		2.9927	3.0697	2.9603
		3.0405	3.0424	2.9653
		2.8015	3.0226	3.0245
		3.0068	3.0762	3.0842
	Mean	**3.0121**	**3.0540**	**3.0319**
	SD	**0.0999**	**0.0190**	**0.0557**
	%CV	**3.32**	**0.62**	**1.84**
	%Bias	**0.40**	**1.80**	**1.06**
QCM	40.00	39.6607	39.4145	39.7755
		39.6343	39.7257	39.6213
		39.6973	39.6583	39.8715
		39.6619	39.5621	39.5043
		39.6263	39.3686	39.7106
		39.8470	39.8130	39.9700
		39.4380	39.8485	39.5269
	Mean	**39.6522**	**39.6272**	**39.7114**
	SD	**0.1204**	**0.1873**	**0.1740**
	%CV	**0.30**	**0.47**	**0.44**
	%Bias	**-0.87**	**-0.93**	**-0.72**
QCH	80.00	81.5956	81.7992	81.3823
		81.4675	81.0396	81.4516
		81.5493	80.6256	81.5115
		81.7911	80.6905	81.3920
		81.4331	81.3350	81.5355
		81.7402	81.2523	81.1105
		80.2187	81.4282	80.4481
	Mean	**81.3994**	**81.1672**	**81.2617**
	SD	**0.5370**	**0.4163**	**0.3851**
	%CV	**0.66**	**0.51**	**0.47**
	%Bias	**1.75**	**1.46**	**1.58**

Table 10 Inter-day precision and accuracy of QC sample

	Back-calculated concentration (ng mL^{-1})			
Run No.	**LLOQ**	**QCL**	**QCM**	**QCH**
	1.00	**3.00**	**40.00**	**80.00**
Run 1	1.0172	3.0777	39.6607	81.5956
	1.0417	3.0877	39.6343	81.4675
	1.0578	3.0775	39.6973	81.5493
	0.9610	2.9927	39.6619	81.7911
	1.0071	3.0405	39.6263	81.4331
	1.0034	2.8015	39.8470	81.7402
	1.0844	3.0068	39.4380	80.2187
Run 2	1.0020	3.0492	39.4145	81.7992
	1.0934	3.0698	39.7257	81.0396
	1.0879	3.0481	39.6583	80.6256
	1.0173	3.0697	39.5621	80.6905
	1.0650	3.0424	39.3686	81.3350
	0.9575	3.0226	39.8130	81.2523
	1.0352	3.0762	39.8485	81.4282
Run 3	1.0244	3.0167	39.7755	81.3823
	1.0172	3.0934	39.6213	81.4516
	1.0172	3.0789	39.8715	81.5115
	1.0156	2.9603	39.5043	81.3920
	1.0132	2.9653	39.7106	81.5355
	1.0232	3.0245	39.9700	81.1105
	1.0098	3.0842	39.5269	80.4481
Mean	**1.0263**	**3.0327**	**39.6636**	**81.2761**
SD	**0.0360**	**0.0659**	**0.1590**	**0.4388**
%CV	**3.50**	**2.17**	**0.40**	**0.54**
%Bias	**2.63**	**1.09**	**-0.84**	**1.60**
% Recovery	**102.63**	**101.09**	**99.16**	**101.60**

Table 11 One-way ANOVA of QC sample.

Level	F_{cal}	F_{crit}	P-value
LLOQ	0.51	3.55	0.61
QCL	0.69	3.55	0.52
QCM	0.49	3.55	0.62
QCH	0.47	3.55	0.63

4.4.5) Limit of detection and limit of quantitation

The LOD was 0.20 ng mL^{-1} at a signal-to-noise ratio of 1:7 and the LOQ was 0.50 ng mL^{-1} at a signal-to-noise ratio of 1:19, as shown in Tables 12–13.

Table 12 LOD and LOQ from calibration curve 3 days.

Calibration curve	slope	intercept
Day1	0.0179	0.0005
Day2	0.0216	0.0005
Day3	0.0212	0.0029
mean	0.0202	0.0013
SD	0.0021	0.0014
LOD_{cal}	0.23	
LOQ_{cal}	0.69	

Table 13 Verification of LOD and LOQ.

Name	Peak Area	Peak Area IS	Peak area Ratio	Cal conc. [ng mL^{-1}]	Bias [%]	S/N	Mean S/N
LOD (0.1 ng mL^{-1})	n.a.	31967.475	n.a.	n.a.	n.a.	n.a.	n.a.
	n.a.	31830.162	n.a.	n.a.	n.a.	n.a.	
	n.a.	32690.630	n.a.	n.a.	n.a.	n.a.	
	n.a.	41206.233	n.a.	n.a.	n.a.	n.a.	
	n.a.	31694.825	n.a.	n.a.	n.a.	n.a.	
LOD (0.2 ng mL^{-1})	223.714	31697.347	0.007	0.27	34.99	8.15	7.87
	234.845	30953.425	0.008	0.29	47.00	8.50	
	231.606	31131.192	0.007	0.29	43.66	7.23	
	272.784	40793.146	0.007	0.25	26.57	9.64	
	219.693	31099.652	0.007	0.27	35.13	5.86	
LOD (0.3 ng mL^{-1})	289.362	29822.907	0.010	0.39	30.02	10.00	10.36
	372.572	39423.455	0.009	0.38	26.20	10.56	
	255.623	28513.556	0.009	0.36	18.86	10.81	
	267.615	29101.611	0.009	0.37	22.35	11.25	
	209.492	23352.040	0.009	0.36	18.95	9.21	
LOQ (0.5 ng mL^{-1})	362.049	27493.308	0.013	0.55	9.49	19.99	19.12
	499.954	37425.894	0.013	0.56	11.21	18.75	
	368.058	29216.922	0.013	0.52	4.30	19.81	
	405.979	31323.856	0.013	0.54	7.60	18.07	
	391.225	29393.484	0.013	0.55	10.77	18.99	
LOQ (0.7 ng mL^{-1})	642.675	35730.987	0.018	0.77	9.45	25.45	25.67
	660.471	37350.801	0.018	0.75	7.48	27.63	
	625.451	34837.639	0.018	0.76	9.24	25.73	
	469.760	26826.520	0.018	0.74	6.37	23.17	
	461.280	26384.175	0.017	0.74	6.19	26.39	
LOQ (0.9 ng mL^{-1})	555.398	25310.488	0.022	0.95	5.09	35.77	41.04
	741.521	33677.747	0.022	0.95	5.47	41.71	
	540.398	25008.559	0.022	0.93	3.40	40.52	
	527.816	24411.880	0.022	0.93	3.47	42.70	
	522.535	23955.499	0.022	0.94	4.43	44.52	

n.a. : not available

4.4.6) Matrix effect

The matrix effect as %Bias value was less than ±6% and the %CV value was less than ±4%. Regarding the peak area of 11-Nor-Δ9-THC-COOH-d_3, the %CV value was less than ±9%, falling within the acceptance criteria at 15%, as shown in Table 14.

Table 14 Matrix effect.

Matrix No.	Back-calculated concentration (ng mL^{-1})			Peak area IS 11-Nor-Δ9-THC-COOH-d3		
	LLOQ 1.00	QCL 3.00	QCH 80.00	LLOQ 1.00	QCL 3.00	QCH 80.00
1	1.0118	3.2033	84.9121	43091.4658	41351.4876	38609.7711
2	1.0650	3.0912	84.9188	43213.0855	51728.9403	38187.4443
3	0.9728	3.1831	84.8517	43939.8527	42686.6499	38239.5505
4	1.0873	3.1556	85.4894	42676.4880	43105.3350	45472.7099
5	1.0361	3.1904	84.8277	42939.0279	43436.0193	46123.0290
6	0.9833	3.1501	82.2080	44035.1657	43093.3439	38240.7568
7	1.0828	3.1472	84.6343	44141.8104	44530.3043	38588.6434
8	1.0281	3.1268	83.9304	51979.6724	41897.9343	38254.8195
9	1.0370	3.1727	82.8621	53095.1086	41579.6860	38643.3713
10	0.9946	3.1895	83.3360	42879.2299	41796.9783	37343.2531
11	1.0308	3.1815	82.8881	44064.3272	48662.2405	37834.3274
12	1.0163	3.2033	82.6227	52212.7035	41036.2743	45641.0374
13	0.9949	3.1989	83.0523	44075.3648	41899.0773	37118.3713
14	0.9752	3.1952	83.6299	42441.0902	41860.1738	37044.8935
15	1.0955	3.1789	82.1108	42262.4909	49448.6395	37079.3434
16	1.0830	3.1729	82.7225	41496.2615	42330.3937	37348.8637
17	1.0461	3.1943	85.3181	41561.7896	42743.8491	36328.4776
18	1.0382	3.2044	83.2664	42934.2997	41976.8584	36686.8623
19	1.0878	3.1712	85.0858	41509.9193	49159.1614	35074.4456
20	1.0804	3.1739	84.9791	41331.7131	41865.9347	34899.5267
21	1.0333	3.1817	83.4560	41623.1303	41731.4680	36220.6071
22	1.0621	3.1645	84.9744	40630.3206	42716.9930	35560.0834
23	1.0693	3.1989	84.7469	40471.5215	52024.5050	44438.6964
24	1.0660	3.1877	83.8250	42772.9382	42026.5253	45076.6528
25	1.0672	3.1827	82.5602	41374.0562	41170.4002	35479.0271
26	1.0757	3.1712	83.8102	41208.8330	40226.3365	35758.8358
27	1.0809	3.1573	82.5601	42451.7410	40701.6712	36569.5473
28	1.0882	3.1329	84.5651	49097.3015	41918.3977	35740.2123
29	1.0420	3.1833	84.2485	49716.3395	52013.6727	34815.1465
30	1.0424	3.1940	84.8318	42194.1357	41895.3513	37850.4447
Mean	**1.0458**	**3.1746**	**83.9075**	**43914.0395**	**43753.8201**	**38342.2917**
SD	**0.0363**	**0.0256**	**1.0385**	**3529.7658**	**3591.3076**	**3391.1906**
%CV	**3.47**	**0.81**	**1.24**	**8.04**	**8.21**	**8.84**
%Bias	**4.58**	**5.82**	**4.88**			

4.4.7) Dilution integrity

A dilution factor at 20 times showed %Bias between -6.24 and 2.74% with %CV in the range of 0.26% and 1.17%, as shown in Table 15.

Table 15 Dilution integrity

Replicate No.	Back-calculated concentration (ng mL^{-1})			
	D1	**D5**	**D10**	**D20**
	100.00	**40.00**	**20.00**	**10.00**
1	98.9748	41.1517	18.8037	9.6569
2	98.4305	40.6867	18.6676	9.5911
3	98.9061	41.2499	18.5739	9.4133
4	98.4555	41.1335	18.8855	9.4054
5	98.8187	41.2591	18.8330	9.5512
Mean	**98.7171**	**41.0962**	**18.7527**	**9.5236**
SD	**0.2564**	**0.2358**	**0.1283**	**0.1110**
%CV	**0.26**	**0.57**	**0.68**	**1.17**
%Bias	**-1.28**	**2.74**	**-6.24**	**-4.76**

4.4.8) Stability

4.4.8.1) Short-term stability

The short-term stability analysis revealed that the %Bias value fell within ±3%. The %CV was found to be less than ±3% (the acceptance criteria was ±15%), as shown in Table 16.

Table 16 Short term stability

Replicate No.	Back-calculated concentration (ng/mL)			
	8 hours		**24 hours**	
	QCL	**QCH**	**QCL**	**QCH**
	3.00	**80.00**	**3.00**	**80.00**
1	2.9630	80.8927	2.9982	82.1536
2	3.0151	81.6660	3.0796	81.8962
3	3.0086	81.9597	3.0251	82.3828
4	2.9680	80.1655	2.9157	82.1897
5	2.9660	81.9214	3.0958	82.5442
Mean	**2.9841**	**81.3211**	**3.0229**	**82.2333**
SD	**0.0254**	**0.7756**	**0.0718**	**0.2454**
%CV	**0.85**	**0.95**	**2.38**	**0.30**
%Bias	**-0.53**	**1.65**	**0.76**	**2.79**

4.4.8.2) Autosampler stability

The autosampler stability analysis revealed that the %Bias value was less than ±4%. The %CV was found to be less than ±3%, as shown in Table 17.

Table 17 Autosampler stability

Replicate No.	Back-calculated concentration (ng/mL)			
	12 hours		24 hours	
	QCL	QCH	QCL	QCH
	3.00	80.00	3.00	80.00
1	3.0639	82.0123	3.0057	82.4979
2	2.9179	82.7039	2.9555	82.5009
3	2.9526	82.7135	3.0446	82.3786
4	3.0613	82.4489	3.0282	82.3348
5	2.9869	81.8698	3.0673	82.4857
Mean	**2.9965**	**82.3497**	**3.0203**	**82.4396**
SD	**0.0651**	**0.3911**	**0.0427**	**0.0775**
%CV	**2.17**	**0.47**	**1.41**	**0.09**
%Bias	**-0.12**	**2.94**	**0.68**	**3.05**

4.4.8.3) Reanalysis stability

The reanalysis stability revealed that the %Bias value was observed to be less than ±1%. The %CV was found to be less than ±3%, as shown in Table 18.

Table 18 Reanalysis stability after stored in 2-8 °C 72 h.

Replicate No.	Back-calculated concentration (ng/mL)	
	QCL	QCH
	3.00	80.00
1	2.9755	79.9676
2	2.9167	79.5036
3	3.0702	80.8864
4	3.0587	80.1442
5	2.9241	80.6897
Mean	**2.9890**	**80.2383**
SD	**0.0725**	**0.5580**
%CV	**2.43**	**0.70**
%Bias	**-0.37**	**0.30**

CHAPTER V
CONCLUSIONS

A detection method for the 11-Nor-Δ9-THC-COOH in urine was successfully developed and validated using online-solid phase extraction coupled with liquid chromatography-mass spectrometry. The developed method was specific for quantifying the 11-Nor-Δ9-THC-COOH in urine. This approach achieved LOD and LOQ values of 0.20 and 1.00 ng/mL, respectively. This approach proved successful for analyzing concentrations ranging from 1.00 to 100.00 ng/mL without interfering peaks. The parameters for characterizing the proposed approach, such as accuracy, precision, and recovery, meet the recognized requirements. Dilution integrity was confirmed for samples analyzed at concentrations greater than the calibration curve's maximum value. The advantages of the analytical approach established by hydrolysis sample preparation with sodium hydroxide in a dry bath incubator are simplicity, cost-effectiveness, and quick execution in preparing the urine sample, with an analysis time of only 10 minutes. This method is accurate, precise, and suitable for analyzing 11-nor-Δ9-THC-COOH in urine samples in trace amounts.

In this research, 11-Nor-Δ9-THC-COOH was successfully studied by the developed method. Thus, further research should be conducted on other varieties, including CBD. To guarantee that the investigation is thorough and advantageous for medical applications.

REFERENCES

[1] N. Korac, D. Vidic, and D. Sutlovic, "Modified QuEChERS extraction and GC-MS analysis of selected cannabinoids from human urine," *Bulletin of the Chemists and Technologists of Bosnia and Herzegovina*, no. 54, 2020, doi: 10.35666/ghtbh.2020.54.07.

[2] W. Zhang *et al.*, "Extraction and Analysis of Tetrahydrocannabinol, A Cannabis Compound in Oral Fluid," *International Journal of Biology*, vol. 9, no. 1, 2016, doi: 10.5539/ijb.v9n1p30.

[3] P. Frei, S. Frauchiger, E. Scheurer, and K. Mercer-Chalmers-Bender, "Quantitative determination of five cannabinoids in blood and urine by gas chromatography tandem mass spectrometry applying automated on-line solid phase extraction," *Drug Test Anal*, vol. 14, no. 7, pp. 1223-1233, Jul 2022, doi: 10.1002/dta.3241.

[4] J. Goncalves *et al.*, "Cannabis and Its Secondary Metabolites: Their Use as Therapeutic Drugs, Toxicological Aspects, and Analytical Determination," *Medicines (Basel)*, vol. 6, no. 1, Feb 23 2019, doi: 10.3390/medicines6010031.

[5] M. o. P. H. BUREAU OF DRUG AND NARCOTIC. Department of Medical Sciences, *Standard Methods for Analysis of Narcotics in Urine*. 2015.

[6] A. Srisubat *et al.*, "Outcomes of THC Enriched in Advanced Staged Cancer Patients," *Journal of the Department of Medical Services.*, vol. 45, pp. 208-214, 2020.

[7] ธ. นิ่มสมบูรณ์, "พิษวิทยาของกัญชา," *Thai Journal of Hospital Pharmacy*, vol. 30, no. 2, pp. 125-136, 2020. (in Thai)

[8] M. o. P. H. Narcotics Control Management Center, *Recommendation of Cannabis treatment and care in Thailand*. 2019.

[9] M. o. p. h. Department of medical services, *Guidance on Cannabis for Medical Use*. 2022.

[10] United Nations Office on Drugs and Crime, World Drug Report 2006, Available from: https://www.unodc.org/pdf/WDR_2006/wdr2006_volume1.pdf.

[11] L. Vlase, D. S. Popa, D. Zaharia, and F. Loghin, "High-throughput toxicological analysis of Δ9-THC and 11-nor-9-carboxy-Δ9-THC by LC/MS/MS," *Romanian Journal of Legal Medicine*, vol. 18, no. 2, pp. 133-140, 2010, doi: 10.4323/rjlm.2010.133.

[12] M. Woodbridge, *MedicainalCannabisBook*. 2019.

[13] M. A. ElSohly, M. M. Radwan, W. Gul, S. Chandra, and A. Galal, "Phytochemistry of Cannabis sativa L," *Prog Chem Org Nat Prod*, vol. 103, pp. 1-36, 2017, doi: 10.1007/978-3-319-45541-9_1.

[14] ระพีพงศ์ สุพรรณไชยมาตย์ และโชยิตา ภาวสุทธิไพศิฐ., "ประโยชน์และโทษที่อาจเกิดขึ้นจากการใช้กัญชาในทางการแพทย์และการเปิดเสรีการใช้กัญชา," *Journal of Health Systems Research*, vol. 12, pp. 71-94, 2018. (in Thai)

[15] N. Kamkaen. and C. Treesak., "Medicinal Cannabis for Cancer," *The Journal of Chulabhorn Royal Academy*, vol. 1, pp. 16-29, 2019.

[16] R. C. Clarke, *Botany of the genus Cannabis*. Haworth Press, Binghamton, NY, 1999.

[17] M. Strzelczyk, M. Lochynska, and M. Chudy, "Systematics and botanical characteristics of industrial hemp Cannabis sativa L," *Journal of Natural Fibers*,

vol. 19, no. 13, pp. 5804-5826, 2022.
[18] J. Klawitter *et al.*, "An Atmospheric Pressure Chemical Ionization MS/MS Assay Using Online Extraction for the Analysis of 11 Cannabinoids and Metabolites in Human Plasma and Urine," *Ther Drug Monit*, vol. 39, no. 5, pp. 556-564, Oct 2017, doi: 10.1097/FTD.0000000000000427.
[19] A. Marston, "Role of advances in chromatographic techniques in phytochemistry," *Phytochemistry*, vol. 68, no. 22-24, pp. 2786-98, Nov-Dec 2007, doi: 10.1016/j.phytochem.2007.08.004.
[20] F. Grotenhermen, "Pharmacokinetics and pharmacodynamics of cannabinoids," *Clin Pharmacokinet*, vol. 42, no. 4, pp. 327-60, 2003, doi: 10.2165/00003088-200342040-00003.
[21] K. Woranisarakul, "Pharmacology of cannabinoids," *J Thai Stroke Soc.*, vol. 20 (3), 2021.
[22] F. Grotenhermen, "Pharmacology of cannabinoids," *Neuro Endocrinol Lett*, vol. 25, no. 1-2, pp. 14-23, Feb-Apr 2004. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/15159677.
[23] S. Chayasirisobhon, "Mechanisms of Action and Pharmacokinetics of Cannabis," *Perm J*, vol. 25, pp. 1-3, Dec 2020, doi: 10.7812/TPP/19.200.
[24] M. A. Huestis, "Human Cannabinoid Pharmacokinetics," *CHEMISTRY & BIODIVERSITY*, vol. 4, pp. 1770-1804, 2007.
[25] M. A. ElSohly, W. Gul, and L. A. Walker, "Pharmacokinetics and Tolerability of Delta9-THC-Hemisuccinate in a Suppository Formulation as an Alternative to Capsules for the Systemic Delivery of Delta9-THC," *Med Cannabis Cannabinoids*, vol. 1, no. 1, pp. 44-53, Jun 2018, doi: 10.1159/000489037.
[26] F. Grotenhermen, "Clinical Pharmacokinetics of Cannabinoids," *Journal of Cannabis Therapeutics*, vol. 3, no. 1, pp. 3-51, 2003, doi: 10.1300/J175v03n01_02.
[27] K. R. Allen, "Screening for drugs of abuse: which matrix, oral fluid or urine?," *Ann Clin Biochem*, vol. 48, no. Pt 6, pp. 531-41, Nov 2011, doi: 10.1258/acb.2011.011116.
[28] ชยันต์ พิชียรสุนทร, แม้นมาส ชวลิต และวิเชียร จีรวงส์. คำอธิบายตำราพระโอสถพระนารายณ์ฉบับเฉลิมพระเกียรติ72 พรรษามหาราชา 5 ธันวาคม พุทธศักราช 2542. พิมพ์ครั้งที่ 2. กรุงเทพฯ : สำนักพิมพ์อมรินทร์และมูลนิธิภูมิปัญญา. 2548. (in Thai)
[29] กรมการแพทย์แผนไทย (2018) https://web.facebook.com/643148052494633/photos/a.644390729037032/1292793800863385/?type=1&theater. (in Thai)
[30] S. Konig, B. Aebi, S. Lanz, M. Gasser, and W. Weinmann, "On-line SPE LC-MS/MS for the quantification of Delta9-tetrahydrocannabinol (THC) and its two major metabolites in human peripheral blood by liquid chromatography tandem mass spectrometry," *Anal Bioanal Chem*, vol. 400, no. 1, pp. 9-16, Apr 2011, doi: 10.1007/s00216-011-4708-x.
[31] S. C. W. de Souza Eller, L. G. Flaiban, B. A. P. B. Paranhos, J. L. da Costa, F. R. Lourenço, and M. Yonamine, "Analysis of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in urine samples by hollow fiber-liquid phase microextraction and gas chromatography–mass spectrometry in consideration of measurement uncertainty," *Forensic Toxicology*, vol. 32, no. 2, pp. 282-291, 2014, doi: 10.1007/s11419-014-0239-4.
[32] S. Golding Fraga, J. Diaz-Flores Estevez, and C. Diaz Romero, "Stability of

cannabinoids in urine in three storage temperatures," An*n Clin Lab Sci*, v*o*l. 28, no. 3, pp. 160-2, May-Jun 1998. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/9646857.

[33] NorthEast BioLab Agile Bioanalysis Services CRO. Complete Guide On LC-MS: Working, Analysis & Methods. Retrieved February 4, 2023, from https://www.nebiolab.com/complete-guide-on-liquid-chromatography-mass-spectrometry-lc-ms/

[34] J. Griffiths, "A brief history of mass spectrometry," An*al Chem*, v*o*l. 80, no. 15, pp. 5678-83, Aug 1 2008, doi: 10.1021/ac8013065.

[35] N. V. Heuett, C. E. Ramirez, A. Fernandez, and P. R. Gardinali, "Analysis of drugs of abuse by online SPE-LC high resolution mass spectrometry: communal assessment of consumption," Sc*ience of the Total Environment*, v*o*l. 511, pp. 319-330, 2015.

[36] Thermo Fisher Scientific. Vanquish Online SPE HPLC and UHPLC Systems. Retrieved February 4, 2023, from https://www.thermofisher.com/th/en/home/industrial/chromatography/liquid-chromatography-lc/hplc-uhplc-systems/vanquish-online-spe-hplcuhplc.html

[37] S. Yoshida e*t al.*, "A bacterium that degrades and assimilates poly(ethylene terephthalate)," Sc*ience*, v*o*l. 351, no. 6278, pp. 1196-9, Mar 11 2016, doi: 10.1126/science.aad6359.

[38] J. Stone, "Sample preparation techniques for mass spectrometry in the clinical laboratory," in Ma*ss Spectrometry for the Clinical Laboratory*, 2017, pp. 37-62.

[39] J. D. Cook, K. A. Strauss, Y. H. Caplan, C. P. Lodico, and D. M. Bush, "Urine pH: the effects of time and temperature after collection," J *Anal Toxicol*, v*o*l. 31, no. 8, pp. 486-96, Oct 2007, doi: 10.1093/jat/31.8.486.

[40] Agilent Technologies. Sample Clean-Up in the Fast Lane. Retrieved February 4, 2023, from https://www.agilent.com/cs/library/eseminars/public/Sample_Clean-Up_in_the_Fast_Lane_04032018%20.pdf.

[41] Published by Oxford University Press, Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Journal of Analytical Toxicology. 2013, 37, 452–474.

[42] U.S. Department of Health and Human Services, Food and Drug Administration. Guidance for industry bioanalytical method validation. [Online]. 2018 [34 screens]. Available from: https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf.

[43] P. Sharma, V. Rao, P. K. Chand, and P. Murthy, "Quantification of Delta9-tetrahydrocannabinol in urine as a marker of cannabis abuse," In*dian J Med Res*, v*o*l. 158, no. 5&6, pp. 535-541, Nov 1 2023, doi: 10.4103/ijmr.IJMR_3899_20.

[44] M. E. Leon-Gonzalez, N. Rosales-Conrado, L. V. Perez-Arribas, and L. M. Polo-Diez, "Large injection volumes in capillary liquid chromatography: Study of the effect of focusing on chromatographic performance," J *Chromatogr A*, v*o*l. 1217, no. 48, pp. 7507-13, Nov 26 2010, doi: 10.1016/j.chroma.2010.09.076.

[45] D. B. Ren, Z. H. Yang, Y. Z. Liang, W. Fan, and Q. Ding, "Effects of injection volume on chromatographic features and resolution in the process of counter-current chromatography," J *Chromatogr A*, v*o*l. 1277, pp. 7-14, Feb 15 2013, doi: 10.1016/j.chroma.2012.12.035.

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